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ACETYLCHOLINESTERASE AND ACETYLCHOLINE RECEPTOR

Midterm Report

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Our study of acetylcholinesterase (AcChE) is based on the view that the β-trimethylammonio substituent of acetylcholine binds at an uncharged subsite, better termed "trimethyl" than "anionic," and would be more specifically explored by uncharged reagents.

AcChE, isolated from Torpedo nobiliana, analyzed fluorimetrically and by Ellman assay, has K_m = 0.056 mM, k_{cat} = 4.0 x 10^3 sec^{-1} in hydrolysis of acetylthiocholine.

1-Bromo-2-[^{14}C]-pinacolone ([^{14}C]-BrPin) inactivates and labels AcChE, and 5-trimethylammonio-2-pentanone (TAP), the isosteric ketone analogue

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19. ABSTRACT (cont.)

of acetylcholine, prevents this inactivation and reduces ^{14}C introduction. Two ^{14}C are introduced per enzyme unit inactivated, and one ^{14}C is excluded per enzyme unit protected by TAP. 3-Trimethylammoniacetonphenone, an aromatic analogue of TAP, protects as effectively as TAP. Other benzene and pyridine derivatives, binding at an aryl subsite, inhibit substrate hydrolysis effectively, but protect less effectively against BrPin inactivation, while reducing ^{14}C introduction.

Noncompetitive inhibition by tetrasubstituted ammonio inhibitors increases with decreasing substrate reactivity, indicating binding to enzyme-substrate complex (ES), in contrast to the effects of trisubstituted analogues.

3-Phenyl-3-trifluoromethyldiazirines have been prepared as photoinactivating agents.

Prior inactivation by BrPin prevents introduction of [^{3}H]-diisopropyl fluorophosphate (DFP) and [^{3}H]-soman. Prior inactivation by DFP does not prevent introduction of ^{14}C from [^{14}C]-BrPin.

A manuscript on inactivation by [^{14}C]-BrPin has been prepared.

FOREWORD

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Saul G Cohen Sept 12 1989

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SUMMARY

This research seeks information about the properties and amino acid content of the active site of acetylcholinesterase (AcChE). It is based on the view that the subsite or cavity at which the positively charged β-substituent of the natural substrate, acetylcholine (AcCh), binds is not anionic, as is generally accepted, but uncharged and complementary to the trimethyl-substituted character of that substituent. The subsite would be more specifically explored by uncharged reagents, while positive reagents may react at surface negative sites. Thus uncharged irreversible and reversible inhibitors and substrates are obtained or synthesized and their reactions with the enzyme are studied. Primary emphasis is placed on labeling the active site with 1-bromo-2-[^{14}C]-pinacolone, $(CH_3)_3C^{14}COCH_2Br$ ([^{14}C]-BrPin); we have found in previous work that BrPin inactivates AcChE with the same kinetics toward hydrolysis of substrates of widely varied structure, and this inactivation is retarded by active-site-directed reversible inhibitors.

A second line of inquiry derives from the view that the conformation of the active site is stabilized in part by interactions of aryl side chains which are contiguous with the trimethyl and esteratic subsites. These aryl groupings provide an additional subsite at which added aryl compounds may bind and, depending on structure, act as reversible inhibitors, irreversible inactivators, labeling agents and reactivators. Derivatives of benzene and pyridine designed to perform these functions are being examined.

For labeling studies, AcChE is isolated from Torpedo nobiliana by affinity chromatography, and characterized by gel electrophoresis and autoradiography after treatment with [^{3}H]-DFP and [^{14}C]-BrPin. Protein and label are concentrated at about 70 kDa. Enzyme content is determined fluorimetrically by burst release of N-methyl-7-hydroxyquinolinium ion (M7HQ), and comprises about 65% of the purified protein. Assay by the Ellman procedure leads to K_m = 0.056 mM, $k_{cat} = 4.0 \times 10^3$ sec^{-1} in hydrolysis of acetylthiocholine (AcSCh). For most purely kinetic studies, Electrophorus AcChE, obtained from Sigma, is used. It has much extraneous, possibly stabilizing, protein and about 16% enzyme of about 50 kDa.

[^{14}C]-BrPin is prepared on a 60-μmole scale by reaction of 1-[^{14}C]-acetyl chloride with a slight (about 3%) deficiency of tert-butylmagnesium chloride in anhydrous ether in the presence of Cu_2Cl_2 catalyst under inert atmosphere, followed by treatment with one equivalent of bromine, and washing to remove Cu^{2+} and residual Br_2, powerful poisons for AcChE. The product solution contains [^{14}C]-pinacolone, [^{14}C]-BrPin, and 1,1-di-

bromo-2-[^{14}C]-pinacolone ([^{14}C]-Br_2Pin) in an approximate ratio of 1:2:1. The K_i values of the three components are 5.5, 0.18, and 0.8 mM, respectively, and the solution inactivates AcChE similarly to commercial BrPin of like concentration. Br_2Pin does not inactivate AcChE at the level at which it is present.

5-Trimethylammonio-2-pentanone, $(CH_3)_3N^+CH_2CH_2CH_2COCH_3$ (TAP), isosteric with AcCh, is an effective reversible inhibitor of AcChE. Its effects on the action of inactivators are studied as diagnostic of their active-site-directed properties. Effects of aromatic inhibitors on action of BrPin and [^{14}C]-BrPin are also studied.

Inactivation of 1-4 μM Torpedo AcChE by 1 mM [^{14}C]-BrPin at pH 7.0, about 50% in 90 min, is prevented by 15 mM TAP; two ^{14}C are introduced per unit inactivated; one ^{14}C is excluded per unit protected. Protection fails on long-term, about 20-hr, incubation. ^{14}C enters denatured enzyme more slowly than into active enzyme. Decamethonium (DEC) protects against inactivation by [^{14}C]-BrPin. [^{14}C]-BrPin enters DFP-inactivated enzyme more slowly than into active enzyme. Phenyltrimethylammonium ion (PTA) offers less protection to purified Torpedo AcChE against [^{14}C]-BrPin than TAP, but appears to protect effectively the more stable Electrophorus AcChE.

Inactivation of Torpedo AcChE by [^{14}C]-BrPin is more rapid at pH 6.0 than at pH 7.0, with 25 % less introduction of ^{14}C, and is prevented by 14 mM TAP. PTA and 3-dimethylamino-N-methylpyridinium ion (DMMP) retard inactivation less efficiently, but exclude about 40% ^{14}C. 3-Trimethylammonioacetophenone (TMAAPh), an aromatic analogue of TAP, protects AcChE against [^{14}C]-BrPin as effectively as TAP. At pH 5.4, inactivation is even more rapid, but denaturation is also rapid. TAP and PTA protect as they do at pH 6.0.

Treatment of Torpedo AcChE with [^{14}C]-BrPin, followed by tryptic digestion, G-50 Sephadex filtration and HPLC separation leads to ^{14}C-labeled peptide of M_r about 1300. Enzyme samples, partially inactivated by [^{14}C]-BrPin at pH 6.0 and protected by TAP, are now being worked up for isolation of the labeled peptides.

Substituted pyridines are being studied as reversible inhibitors, protecting agents, and inactivating agents. Reversible inhibition of Electrophorus AcChE is favored by electron-withdrawing acetyl and nitro substituents; by alkyl, alkoxy and amino groups; very strongly by dimethylamino substituents and by N-methylation, and is unaffected by conversion to the N-oxide. It is increased by protonation of the pyridines at low pH. N-Methylpyridinium and DMMP offer only weak protection against inactivation of Torpedo AcChE by [^{14}C]-BrPin.

Competitive and noncompetitive inhibition of hydrolysis by Electrophorus AcChE of four substrates, AcCh; 2-N,N-diethyl-N-butylammonioethyl acetate; 3,3-dimethylbutyl acetate; methylsulfonylethyl acetate and n-butyl acetate, by six pairs of quaternary and tertiary ammonio inhibitors, namely, tetramethyl and trimethylammonium ions; choline and dimethylammonioethanol and their ethyl ethers, 5-trimethyl- and 5-dimethylammonio-2-pentanone; 2-acetamidoethyltrimethylammonium iodide and 2-acetamidoethyldimethylammonium chloride; and tert-butyl- and isopropylammonium ions. Tetrasubstituted compounds show more favorable noncompetitive inhibition with slower substrates, indicating binding to enzyme-substrate complex, ES, forming ESI, rather than to acetylenzyme, EA. By extension, inhibition by high concentration of substrate, AcCh, is attributed to formation of ESS, not EAS.

3-Phenyl-3-trifluoromethyldiazirine and 3-(3'-iodo)phenyl-3-trifluoromethyldiazirine have been prepared as potential photoinactivating agents for acetylcholine receptor and AcChE.

A manuscript, "Reactions of 1-Bromo-2-^{14}C-Pinacolone with Acetylcholinesterase from Torpedo nobiliana. Effects of 4-Trimethylammonio-2-pentanone and Diisopropyl Fluorophosphate," has been prepared for publication.

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(1) Statement of the Problem Under Study

A. The Trimethyl Site

Acetylcholinesterase (AcChE) has been widely studied for many years because of its great importance in regulating neurotransmission. This study led to the serine-hydroxyl as an essential group in the active site of many hydrolytic enzymes. While other serine enzymes have been crystallized and their active sites described in some detail, and the linear sequence of AcChE has recently been established, no other amino acid has been unequivocally characterized in the active site of AcChE.

It has long been accepted that the active site of AcChE has an anionic subsite to which the cationic group of the substrate, acetylcholine (AcCh), is attracted, and this has guided study and interpretation. From study of varied substrates and inhibitors, we have concluded that the binding subsite is uncharged, "trimethyl" rather than "anionic," and that the observed effects of anionic charge are accounted for by the excess negative charges on the enzyme surface, peripheral to the active site, arising in a protein of isoelectric point about pH 5, which is active and studied at pH 7-8. We propose that the active site would be more specifically studied by uncharged reagents and have chosen 1-bromopinacolone, $(CH_3)_3C-COCH_2Br$ (BrPin) as such an alkylating reagent, with its tert-butyl group complementary to the trimethyl subsite. We prepare BrPin labeled with ^{14}C and inactivate the enzyme with it, and are engaged in degrading the enzyme to identify the modified active site amino acid. We have prepared and surveyed other uncharged compounds, such as chloromethyl pivalate, $(CH_3)_3CCO_2-CH_2Cl$, and methyl methanesulfonate, $CH_3SO_2OCH_3$, which have potential use, but BrPin is our reagent of choice.

B. Noncompetitive Inhibition

Noncompetitive inhibition of AcChE by substituted ammonium ions and by substrate is generally accepted as arising from binding to the acetylenzyme intermediate, EA, forming EAI or EAS of low or no reactivity. This view has arisen largely from study of tri-substituted ammonium ions, and extension to the substrate seems questionable. This leads us to comparison of a series of homologous tri- and tetra-substituted reversible inhibitors, structurally related to the natural substrate, AcCh, and to the view that noncompetitive inhibition by such compounds arises from their binding to the enzyme-substrate complex, ES, forming ESI and ESS.

C. The Aryl Site

Study of derivatives of benzene and phenol as reversible inhibitors of AcChE has led us to the view that the active site includes an aryl binding subsite, contiguous with the trimethyl and esteratic subsites, possibly comprising aromatic amino acid side chains. This led us to consider uncharged aromatic alkylating agents, bromoacetyl derivatives of benzene and pyridine, as possible reagents to label amino acids at or near this aryl subsite. These studies may lead to potentially superior medicinal reagents acting from this subsite.

(2) Background and Review of Appropriate Literature

A. The Trimethyl Site

(I) Substrates

The structural features involved in the interactions of AcCh, $(CH_3)_3N^+CH_2CH_2OCOCH_3$, with AcChE are the three N-methyl groups, the positive charge, and the ester group. The part of the active site of AcChE at which the trimethylammonium group binds has generally been depicted as anionic (1-3), with its negative charge increasing enzymic activity by attracting, binding and orienting cationic substrates (4). It has been noted that successive N-methylation of alkylammonium ions, starting with methylamine and ethanolamine, improves binding, and N-methylation of substrates, starting with β-aminoethyl acetate, increases both binding and reactivity, and the N-methyl groups contribute more to binding than does coulombic attraction (4-6). Thus it has been proposed that less than 10% of the binding of tetraalkylammonium ions is due to their charge and that there is no negative charge in the purported anionic binding site (7). However, this calculation did not take into account the decrease in solvation of ammonium ions with increasing N-methylation (5), and the presence of a negative charge in the purported anionic site remains widely accepted (8).

Addition of methyl substituents in uncharged analogues of the β-ammonioethyl acetate, from ethyl propionate to 3,3-dimethylbutyl acetate, $(CH_3)_3CCH_2CH_2OCOCH_3$ (DMBAc), the carbon analogue of AcCh, also led to progressively increasing reactivity toward AcChE (1,9). Thus the rate constant for reaction of DMBAc with AcChE is very high, 1-2 x 10^6 $M^{-1}sec^{-1}$ (10-11), though somewhat lower than that for AcCh, 5 x 10^7 $M^{-1}sec^{-1}$ (11).

The β-ammonio substituents increase the intrinsic hydrolytic reactivity of such esters toward hydrolysis by hydroxide, as compared with the uncharged carbon analogues. We have

deemed it appropriate to apply normalization factors to the enzymic hydrolysis rates, as has been done in studies of chymotrypsin, which also reacts via an acyl-serine intermediate (12). This was done in the study of electrophorus AcChE for a series of 14 β-substituted ethyl acetates, $X\text{-}CH_2CH_2OCOCH_3$, with X = the four ammonio groups, H_3N^+ to $(CH_3)_3N^+$, the analogous carbon-centered substituents, CH_3 to $(CH_3)_3C$, and CH_3O, HO, Cl, Br, N≡C and I (11). The normalization factors were the ratios of the base-catalyzed hydrolysis rate constant for AcCh to the corresponding rate constants for each of the substrates. We then found that the normalized enzymic reactivities [$\log(k_{2(n)}/K_s)$, where $k_{2(n)}$ is the normalized acylation rate constant and K_s is the binding constant] for these compounds, were proportional to the apparent molar volumes (V_{25}) of the β-substituents, X (Eq. 1).

$$\log(k_{2(n)}/K_s) = a\,V_{25} + C \qquad (1)$$

The normalization factor applies to the acylation rate constant, k_2. Since the reaction rate may be described by either k_2/K_s or k_{cat}/K_m, we have also applied the factor to the latter ratio. In a later study (13) of another set of substrates (Table I), this normalized substrate reactivity was also found to correlate with volume of uncharged and cationic β-substituents as measured by molar refraction (MR) (14-17). Reactivity increased to and leveled at β-$(CH_3)_3C$- and β-$(CH_3)_3N^+$-, while the trimethyl site accommodated the larger $(CH_3)_3Si$- group, with similar reactivity. β-Substituents with polar surfaces, sulfone, $CH_3S(O_2)$-, amine oxide, $(CH_3)_2N^+(O^-)$-, and sulfoxide, $CH_3S(O)$-, led to lower reactivity than consistent with MR values, possibly because they do not remove water from the active site; however, they led to much higher reactivity than consistent with hydrophobicity (Π), discussed below, as do the cationic substituents (13).

The enzymic reactivity of these acetate esters of widely varied structure was accounted for by two factors: (i) their intrinsic hydroxide-catalyzed reactivity and (ii) the volume of the β-substituent and thus its fit into the active site, and the resulting effect on placing the ester group at the esteratic site. K_s values for AcCh and DMBAc are similar, indicating no substantial coulombic effect, and the effects of charge on binding in the other cationic and uncharged pairs of substrates appeared even smaller (11).

The effect of the positive charge in AcCh is to increase the rate of its hydroxide-catalyzed hydrolysis over that of DMBAc by a factor of about 40 (2.8/0.07, Table I), and the normalized reactivities are quite similar. No specific effect of anionic charge on the enzymic hydrolysis rate and thus no evidence for an anionic site is observed. On this basis we

propose that the part of the active site at which the trimethylammonio group of AcCh and the β-substituent of other acetate substrates bind is not anionic, and is more accurately considered trimethyl, complementary to this character of the β-substituent of AcCh rather than to its positive charge.

Others have noted a relation between enzymic reactivity and the hydrophobicity (Π) of the β-substituents of alkyl esters IIA, IIIA, and VA (Table I) (10). Hydrophobicity is a parameter derived from 2-octanol/water distribution coefficients; it is related to capacity for hydrophobic interactions and is used in drug design (18). For these hydrocarbon substituents, values of Π are proportional to volume, but use of this parameter, Π, does not allow the non-polar, the dipolar and the water-soluble ammonio derivatives to be correlated on the same scale. The correlation with volume does allow the reactivity of the varied classes of substrates to be so correlated.

(II) Reversible Inhibitors

We have also examined the effect of positive charge on binding of reversible inhibitors (19). The enzyme has isoelectric point of about pH 5 (20-21) and thus excess negative charge on its surface at pH 7-8. Cationic reversible inhibitors structurally related to AcCh do bind better than their uncharged analogues by small factors, corresponding to about 1 kcal/mole of binding energy, much less than would be caused by interaction of $(CH_3)_3N^+$ with a "contact" anionic O^- that is implied by a specific anionic site (19). Also, ionic strength effects on binding and on hydrolysis have been interpreted in terms of multiple anionic charges on the enzyme surface (22), but they may not be within the cavity which envelops the β-tert-butyl or trimethylammonio substituent.

Each of the inhibitors which we studied, structurally related to AcCh, whether neutral or cationic, showed essentially the same binding constant when retarding hydrolysis of AcCh and its uncharged analogue, DMBAc. This indicated that the β-trimethylammonio and β-tert-butyl groups of the two substrates and of the related inhibitors bound at the same subsite (19). The substrate study had indicated that this subsite is uncharged trimethyl, and the nature of this site could then be explored more specifically with uncharged reagents (12). Indeed, arylaziridinium reagents, intended to alkylate the anionic site and prevent substrate access, completely inhibited hydrolysis of cationic, but not of neutral, esters (23-24). We took this to indicate not that there are separate anionic and neutral subsites but that cationic, irreversible inhibitors may react with peripheral anionic groups, increasing positive charge and repelling cationic substrates. They modify the active site domain but allow uncharged substrates to bind at the one

trimethyl site and react at reduced, but very substantial, rates.

Effects of charge, volume and surface properties on binding of inhibitors at the trimethyl site, supplementing our study on substrates, have also been examined (Table II) (25). tert-Butyl, $(CH_3)_3C$, trimethylsilyl, $(CH_3)_3Si$, and methylsulfonyl, $CH_3S(O_2)$, substituents lead to similar bindings; trimethylammonio, $(CH_3)_3N^+$, and dimethylsulfonio, $(CH_3)_2S^+$, substituents lead to similar, but stronger bindings. As fourth substituents, $-CH_3$ is less effective than $-CH_2CH_2OH$ and $-CH_2CH_2OCOCH_3$ which have similar effect. Positive charge increases binding by 1.5 kcal/mole.

Sterically similar alcohols with tetra-substituted uncharged β-groups, $(CH_3)_3SiCH_2CH_2OH$, $(CH_3)_3CCH_2CH_2OH$ and $CH_3S(O_2)CH_2CH_2OH$, bind similarly, K_i = 3-9 mM, and the binding of each is similar to that of the corresponding acetate substrate. Cationic analogues, $(CH_3)_3N^+CH_2CH_2OH$ and $(CH_3)_2S^+CH_2CH_2OH$, also bind in a similar manner, K_i = 0.4 mM, similar to the K_m values of their acetate substrates, and they bind more strongly than the uncharged alcohols by about 1.5 kcal/mole, attributable to the multiple surface anionic charges (22).

Although the $-COCH_3$ group in esters with strongly binding β-substituents makes essentially no contribution to binding over that of the alcohols, in esters with weakly bound β-substituents, $(CH_3)_2N^+(O^-)$, $CH_3N^+H_2$, $CH_3S(O)$, CH_3CH_2, and CH_3S, binding is dominated by the ester $-COCH_3$ group; these esters have K_m values about 16 mM (25).

In comparisons of $CH_3S(O_2)CH_2CH_2OH$ with $CH_3SO_2CH_3$, $(CH_3)_2S^+CH_2CH_2OH$ with $(CH_3)_3S^+$, and choline with $(CH_3)_4N^+$, hydroxyethyl leads to more favorable binding than methyl by about 0.8 kcal/mole, despite lower hydrophobicity. Two hydrophobic methyl groups (comparison of 3,3-dimethylbutanol with n-butanol) and two hydrophilic sulfone O atoms, (comparison of 2-methylsulfonylethanol with 2-methylthioethanol), each increases binding similarly, by 1.0 kcal/mole (25).

In comparison of $(CH_3)_2SO_2$ with $(CH_3)_3S^+O$, replacement of O by CH_3 and introduction of a positive charge increases binding by 1.8 kcal/mole. Conversion of $(CH_3)_3S^+$ to $(CH_3)_3S^+O$ has only a small effect on binding. There is no change in charge, equivalence of CH_3 and O in binding is seen again, and binding remains high. However, $(CH_3)_3N^+O^-$ does not bind to AcChE, and conversion of 1-dimethylammonio-4-pentanone and 2-dimethylammonioethyl acetate to their N-oxides, changes of $\rightarrow N^+H$ to $\rightarrow N^+(O^-)$, decrease binding by 1.5 kcal/mole, as a positive charge is replaced by a strongly hydrophilic dipolar bond (25).

In results reported (26) but not published, trichloroethanol, Cl_3CCH_2OH, $K_{i(com)}$ = 42 mM, $K_{i(nonc)}$ = 110 mM,

inhibits similarly to its trimethyl analogue, neopentyl alcohol, $(CH_3)_3CCH_2OH$, $K_{i(com)}$ = 29 mM, $K_{i(nonc)}$ = 74 mM, and to the ether isomer of the latter, tert-butyl methyl ether, $(CH_3)_3COCH_3$, $K_{i(com)}$ = 25 mM, $K_{i(nonc)}$ = 24 mM. Chloral hydrate, $Cl_3CCH{=}O{\cdot}H_2O$, is an effective competitive reversible inhibitor, K_i = 1 mM, binding substantially more strongly than its trimethyl analogue, pivalaldehyde, $(CH_3)_3CCH{=}O$, K_i = 10 mM.

(III) Irreversible Inhibitors

Our view of the purported anionic site as trimethyl was borne out in studies with BrPin (27). This uncharged reagent inhibited AcChE irreversibly. The same rate for inhibition of hydrolysis was observed with a variety of both cationic and neutral substrates, and its inhibiting reaction was retarded by reversible quaternary and trimethylammonio inhibitors to an extent appropriate to their binding constants. These results indicate that both classes of compounds bind at a single trimethyl site (27). BrPin is being used to label the active site.

Compounds structurally related to the other substrates and reversible inhibitors, referred to above, were devised and studied as irreversible inactivators (26): methyl methanesulfonate, $CH_3SO_2OCH_3$, K_i = 96 mM, at 9 mM inactivated Electrophorus AcChE 40% in 1 hr, completely in 4 hr, in a process retarded by tetramethylammonium ions. Methylsulfonyl chloride, CH_3SO_2Cl, at 3 mM appeared as active as the fluoride, leading to complete inactivation in 10 min; at 0.3 mM, to 50% inactivation in 10 min; at 0.03 mM, to 38% in 10 min. Chloromethyl pivalate, $(CH_3)_3CCO_2CH_2Cl$, chloromethyl acetate, $CH_3CO_2CH_2Cl$, and trimethylammoniomethyl acetate, the lower homologue of AcCh, were moderately reactive substrates and, leading to formaldehyde in the active site after hydrolysis, led to partial inactivation. tert-Butyl peracetate, $CH_3COO_2C(CH_3)_3$, was hydrolyzed, $k_{cat}/K_m = 1.2 \times 10^6\ M^{-1}sec^{-1}$, a rate similar to those of the preceding compounds and of DMBAc. Leading to tert-butyl hydroperoxide in the active site, it led to partial inactivation, and did so more effectively than did comparable incubation with the hydroperoxide introduced in the medium (26). Similarity in rates despite shorter span than DMBAc may arise from higher intrinsic hydrolytic reactivity, hydroperoxide being a better leaving group than alkoxide.

B. Noncompetitive Inhibition

Noncompetitive inhibition of AcChE by substituted ammonium ions, and this inhibition by substrate, are accepted as arising from binding to the acetylenzyme intermediate, EA, forming EAI or EAS of low or no reactivity; binding to the initial enzyme-substrate complex, ES, forming ESI and ESS, has been thought

not to occur (28-30). This view probably originated from the concept of an ionic site, viewed as the (one) anionic site, free in EA, occupied in ES. It then gained support from high noncompetitive components in inhibition by tertiary ammonium ions, i.e., $(CH_3)_3N^+H$, etc., in the rapid hydrolysis of AcCh, in which much EA is present, and decreasing noncompetitive components in hydrolysis of less reactive substrates, in which little EA and much ES is present in the steady state. More recent results, indicating that there are multiple anionic charges in the vicinity of the active site (20,22), and that there is no anionic charge in the cavity in which the cationic group of AcCh binds, which we term trimethyl (11), call this accepted interpretation into question. Study of noncompetitive inhibition of substrates of varying enzymic reactivity by tetra-substituted compounds, i.e., $(CH_3)_4N^+$, etc., more closely related to the natural substrate, and thus to inhibition by the substrate itself, seemed to us to merit examination.

C. The Aryl Binding Site

(I) Substituted Benzenes and Phenols

Cationic aromatic compounds have long been known to affect AcChE. These include the carbamylating toxic alkaloid physostigmine (31), the related synthetic reagent neostigmine (32), and the reversible inhibitors phenyltrimethylammonium ion (PTA) (K_i = 0.04 mM) (33) and 3-trimethylammoniophenol (TMAP) (K_i = 0.0003 mM) (33). It seemed of interest to us, by analogy with our studies of uncharged aliphatic substrates and inhibitors, to study substituent effects in uncharged aromatic compounds. Such studies have been reported in some inhibitions of AcChE, and the Hammett equation, $\log k/k_o = \sigma\rho$, was applied (34-37). This equation has been used to correlate the effects of polar substituents on the rates and equilibria of many reactions of aromatic compounds. Each substituent is characterized by a value, σ, a measure of its electron-withdrawing or donating property, a positive value indicating electron-withdrawal, negative value indicating electron-donation, and higher absolute values corresponding to greater effects. Each reaction is characterized by a value, ρ, that for the ionization of benzoic acids having value 1, positive values indicating processes increased by electron withdrawal, and negative values for processes increased by electron-donation. For inactivation of AcChE by a limited series of phenyl N-methylcarbamates, a Hammett plot led to negative ρ (34); for substituted-benzyl N-methylcarbamates, in contrast, the nitro group led to strong binding to AcChE (35); in hydrolysis of substituted-phenyl phosphates, positive ρ was observed and attributed to leaving group activity (36); and in hydrolysis of substituted-phenyl acetates, greatest binding and reactivity were observed at σ value of about 0 (37).

Since substituent effects in these multi-step reactions are complex, we have examined systematically reversible inhibition by substituted benzenes and phenols. We find three effects which determine binding energies of a wide range of such inhibitors. (i) Electron-attracting substituents greatly increase binding, for example, by a factor of 100 in K_i a series of 11 compounds, from $K_{i(com)}$ = 50 mM for phenol and aniline to 0.46 mM for 4-nitroacetanilide; (ii) dimethylamino and tert-butyl substituents increase binding by interaction, apparently, at the trimethyl subsite; and (iii) the latter interaction increases binding synergistically in meta-substituted phenols. The strong binding constants of PTA and TMAP are accurately accounted for and may be calculated from parameters determined in the binding of uncharged compounds (38) with no additional effect of the positive charge such as purported coulombic interaction with an anionic group. It is noteworthy that nitrobenzene, an effective reversible inhibitor for hydrolysis of AcCh and its uncharged analogue, MBAc, K_i = 0.8 mM, accelerates hydrolysis of ethyl acetate (39), much as small cations do (39). Acetophenone also shows this acceleration.

(II) Pyridines (26)

The existence of an aromatic binding site as an integral part of the active site, and the effective binding of uncharged benzene derivatives, led us to a study of derivatives of pyridine as materials with potentially useful properties (Table III). Non-polar substituents, 4-tert-butyl, 2- and 4-vinyl and 2-oxiryl, with $K_{i(com)}$ values of 1-4 mM, improve binding over that of pyridine by about 1 kcal/mole, possibly by interaction at the trimethyl site. Increase due to the acetyl substituent, about 1 kcal/mole, may arise from its electronegativity, and is similar to that in the acetophenone-benzene pair. Dimethylaminopyridines, interacting with the trimethyl site, bind strongly, by 3 kcal/mole more than pyridine, and by 1.5 to 2 kcal/mole more than aminopyridines. Positively charged resonance contributions are important to this binding. Remarkably, dimethylaminopyridines may bind as well as the permanently cationic N-methylpyridinium ion (26), and may provide permeability through lipid membrane via their uncharged forms.

(III) Irreversible Inhibitors (25)

Phenacyl bromide, $C_6H_5COCH_2Br$, is an inactivator and potential labeling agent at the aryl binding site. At 3 mM, it led to 21% inactivation of Electrophorus AcChE in 2 hr, 32% in 3 hr, 55% in 4.2 hr, 73% in 5.25 hr and complete inactivation when incubated overnight.

Styrene oxide, a weak reversible inhibitor (K_i = 10 mM), is a moderately effective inactivator and also has potential as a labeling agent at the aryl site. At 3 mM, it caused progressive inactivation of Electrophorus AcChE, 21, 46, 69 and 100% inactivation after incubation for 1.0, 3.0, 4.5, and 22 hr, respectively. Inactivation by styrene oxide is retarded by 5-trimethylammonio-2-pentanone (TAP). 2-Pyridyloxirane, K_i = 1.5 mM, binds more strongly to Electrophorus AcChE than its carbon analogue, above, but does not appear to be an inactivator.

Methyl benzenesulfonate, $C_6H_5SO_2OCH_3$ (MBS), at 3 mM, led to 65% inactivation of Electrophorus AcChE in 1 hr and 95% in 2 hr, largely unaffected by 30 mM tetramethylammonium ion. The active site accommodates MBS and $(CH_3)_4N^+$ simultaneously. Inactivation by MBS is retarded by TAP.

Inactivation of Torpedo AcChE by the uncharged styrene oxide and MBS and by the cationic N,N-dimethyl-2-phenyl-aziridinium ion (24) prevented or greatly reduced subsequent introduction of 1,3-[^{3}H]-diisopropyl fluorophosphate ([^{3}H]-DFP) (26). Inactivation of Torpedo AcChE by 1 mM [^{14}C]-BrPin was effectively retarded by 17 mM PTA, with reduced introduction of ^{14}C. However, 4 mM nitrobenzene led to only small retardation of this inactivation (26). Formic acid cleavage of Torpedo enzyme inactivated by [^{3}H]-DFP or by [^{14}C]-BrPin led to radioactivity in the 50 kDa fragment (26). A 1.5 x 170 cm G-50 Sephadex gel filtration column was assembled and successfully separated a single peptide fraction from a trypsin digestion of [^{3}H]-DFP-labeled AcChE of M_r about 1300, containing the radioactivity.

(3) Rationale for the Current Study

(I) BrPin has properties of an active-site-directed inactivator, being equally effective against cationic, uncharged and aromatic substrates, and its own action is retarded by substrate-related reversible inhibitors (27). [^{14}C]-BrPin is being used as a radioactive label to characterize the amino acid residue(s) in the active site. No residue has been unequivocally labeled in the active site other than serine-200. The enzyme is isolated from Torpedo nobiliana, purified and inactivated by [^{14}C]-BrPin (in the presence and absence of reversible inhibitors) and cleaved into fragments which will be characterized and sequenced. Electrophorus AcChE as obtained from Sigma is associated with much extraneous protein; it is convenient and suitable for kinetic studies but not feasibly purified for labeling studies.

(II) Enzyme content of affinity purified protein is determined fluorimetrically so that value of k_{cat} for AcChE from T. nobiliana can be determined, allowing calculation of

the number of ^{14}C introduced per enzyme unit inactivated by [^{14}C]-BrPin.

(III) Noncompetitive inhibition is studied for evidence for formation of ESI and ESS complexes, this may have relevance to the observed introduction of two ^{14}C per enzyme unit inactivated by [^{14}C]-BrPin, the result obtained from (II) above, and to design of reversible inhibitors which may further reduce introduction of ^{14}C.

(IV) It was thought that information about mechanism of inactivation by BrFin might be obtained by study of effects of pH on the inactivation by BrPin, especially at low pH.

(V) Study of substituted benzenes and pyridines indicates that they may be effective reversible inhibitors in the absence of permanent positive charge. This may impart superior permeating properties, combined in the pyridines with increased water solubility. Preparation of uncharged benzene- and pyridine-derived labeling and reactivating reagents will be explored.

(4) Experimental Methods

A. Isolation of AcChE from Torpedo nobiliana

Frozen electric organ of Torpedo nobiliana was obtained from Biofish Associates (Gloucester, MA). Literature procedures (21,40) for AcChE isolation were adapted, with purification by affinity chromatography (41-42). The affinity ligand, N-(6-aminohexanoyl-m-aminophenyl)trimethylammonium bromide hydrobromide, was prepared and was coupled to 6-aminohexanoic acid-Sepharose B (41-42) prior to this study. Details of the ligand preparation and of the tryptic digestion protocol for the isolation of 11S AcChE are to be found in the 1985 Annual Report (43). The enzyme activity was measured by the Ellman assay (44) and the purified AcChE was examined by sodium dodecyl sufate (SDS)-polyacrylamide gel electrophoresis (PAGE) (45-46) on 8% acrylamide slab gel at 100 V. Further details are to be found in the 1985 Annual Report (43).

B. Fluorimetric Assay (47-49)

Active site concentration of AcChE was determined by titration with the carbamoylating agent, N-methyl-(7-dimethylcarbamoxy)quinolinium ion (M7CQ) (47-48). Fluorescence was measured on a Hitachi Perkin Elmer MPF-4 spectrofluorimeter.

C. [^{14}C]-BrPin Synthesis

[^{14}C]-BrPin was prepared on a 60-μmole scale by treatment of $CH_3{}^{14}COCl$ with $(CH_3)_3CMgCl$ in ether, followed by reaction

with bromine. Details of the synthesis of [^{14}C]-BrPin are described in previous reports (26,43,50-51). Freshly distilled $CH_3{}^{14}COCl$ is required, and Cu_2Cl_2 catalysis is required for the reaction of $CH_3{}^{14}COCl$ with $(CH_3)_3CMgCl$. Traces of oxygen cause this reaction to fail, and traces of moisture cause the bromination of $(CH_3)_3C{}^{14}COCH_3$ to fail. A small excess of CH_3COCl is tolerated but a large excess and any excess of Grignard reagent are not. A 1:2:1 mixture of pinacolone and mono- and dibromopinacolones, suitable for labeling studies, is obtained. The yield of BrPin is relatively insensitive to the amount of bromine used; less than one equivalent leads to less conversion of pinacolone to BrPin, more than one equivalent to more conversion of pinacolone to BrPin and more conversion of BrPin to Br_2Pin, with the yield of BrPin relatively unchanged. Pinacolone is not an irreversible inhibitor and Br_2Pin caused no inactivation in the concentration present in the product mixture.

During much of the period covered by this report, $CH_3{}^{14}COCl$, 16.7 mCi/mmole, was obtained fairly reliably from New England Nuclear (Boston, MA). Recently some shipments were useless or poor. We now purchase their standard 57 mCi/mmole material and dilute it with Aldrich Gold Label CH_3COCl. We calculate the specific activity by GLC analysis and scintillation counting of the product. We continue to experience some inconsistencies.

A Shimadzu GC-8AFI gas chromatograph was purchased and will be devoted to analysis of radioactive labeling agents in work on this contract.

D. Kinetic Procedures

Our kinetics studies have largely been carried out with pH Stat-Titrimeter equipment as described in our previous publications (11,19). This equipment is no longer functioning satisfactorily. We have purchased replacement equipment: TTT80-1 Radiometer pH Stat Titrator, ABU 80-2 Radiometer Autoburette with B290 2.5/.25 mL Burette assembly, 99051 pH Stat Interface Module, cables, PHM 82-2 Radiometer Laboratory pH meter, and 38-540 Kipp and Zonen chart recorder.

E. Gel Filtration Chromatography and HPLC

A G-50 Sephadex gel filtration column, 1.5 x 170 cm, was assembled for separation of peptide fractions from tryptic digestion of [^{14}C]-BrPin-inactivated AcChE.

A Rainin HPLC gradient analytical system, comprising a Dynamax 300 Å C-18 column, MacIntosh gradient conversion program, Rainin Rabbit HP drive module, and pump heads, was purchased and is in working order. Four peptides, oxidized glutathione, m.w. 612; bradykinin, m.w. 1240; ACTH, m.w. 2,933;

and somatostatin, m.w. 1638 were first put through individually and then together in a 20-μL solution, and separation was achieved. The gradient was water-0.1% trifluoroacetic acid (TFA) to 1:1 water:acetonitrile-0.1% TFA, running time 30 min, 1.0 mL/min, and protein was observed at 219 nm.

F. Chemicals

Purchased chemicals had physical properties corresponding to literature values or were brought to that state by distillation or crystallization.

N,N-Dimethylcarbamylcholine, acetylcholine chloride, 4-tert-butylpyridine, 2-vinylpyridine, 4-vinylpyridine, 3-acetylpyridine, 2-aminopyridine, 3-aminopyridine, 2-amino-4-methylpyridine, 2-dimethylaminopyridine, 4-dimethylaminopyridine, 3-aminoacetophenone, 2-diethylaminoethanol, choline chloride, n-butyl acetate, isopropylamine, methyl iodide, β-bromoethyl ethyl ether, trifluoroacetophenone, phenacyl bromide, phenylglyoxal monohydrate, chloromethylpyridine, 2-methoxypyridine, acetyl chloride and bromopinacolone were from Aldrich Chemical Co. (Milwaukee, WI). Tetramethylammonium chloride and 25% aqueous dimethylamine were from Eastman Kodak Co. (Rochester, NY). Trimethylammonium chloride was from Fisher Scientific (Medford, MA). Pyridine was from J. T. Baker (from VWR Scientific, Boston, MA). 5-Dimethylamino-2-pentanone was from Sapon Laboratories (from Overlook Industries, Bloomsbury, NJ). 4-N-Nitropyridine-N-oxide was from Pfaltz & Bauer (Waterbury, CT). N-Methyl-(7-dimethylcarbamoxy)quinolium ion (M7CQ) and N-methyl-7-hydroxyquinolium ion (M7HQ) were from Molecular Probes, Inc. (Eugene, OR).

TAP, DMBAc, 2-methylsulfonylethyl acetate, n-butyl acetate, N,N-dimethylamino-2-ethoxyethane, and N,N,N-trimethylammonio-2-ethoxyethane iodide were available from previous studies (13,19,52).

2-Oxyrilpyridine was prepared from 2-vinylpyridine by treatment with N-bromosuccinimide in aqueous dioxane containing acetic acid, followed by treatment with sodium carbonate., bp 46-52°C/0.15-0.25 mm, lit. 58°C/0.2 mm (53).

Pyridine-N-oxide hydrochloride was prepared from pyridine N-oxide by treatment with aqueous HCl and repeatedly recrystallized from isopropyl alcohol, mp 181-182°C, lit. 179.5-181°C (54).

3-Dimethylaminopyridine was prepared by treatment of 3-aminopyridine with formaldehyde and formic acid, bp 66°C/1.6 mm, lit. 58-60°C/0.05mm (55).

N-methylpyridinium iodide (NMP) was prepared from pyridine and methyl iodide in ethanol, mp 118-120°C, lit. mp 117°C (56).

3-Dimethylamino-N-methylpyridinium iodide was prepared similarly from 3-dimethylaminopyridine.

3-Bromoacetylpyridine hydrobromide was prepared by bromination of 3-acetylpyridine in hydrobromic acid, mp 196-198°C, lit. mp 198°C (57).

4-Nitropyridine was prepared in low yield by treatment of 4-nitropyridine-N-oxide with PCl_3. This is a complex reaction leading to 4-chloropyridine and other products (58).

3-Trimethylammonioacetophenone iodide was prepared by treatment of 3-aminoacetophenone with excess methyl iodide in methanol in the presence of potassium carbonate, mp 254°C, from methanol-water, with appropriate NMR spectrum. Attempts to follow the literature procedure without added potassium carbonate failed in our hands, and apparently had led to a less pure product, lit. mp 233-235°C (59).

Triethylene glycol di-tert-butylether is being prepared from the glycol via the di-p-toluenesulfonate, mp 80-81°C from methanol, lit. 80-81°C (60).

2-N,N-Diethylaminoethyl acetate hydrochloride was prepared by treatment of diethylaminoethanol with acetyl chloride (61); the base was freed by aqueous alkali and ether extraction, and distilled, then heated in n-butyl iodide at 80°C for 8 hr, leading to 2-N,N-diethyl-N-butylammonioethyl acetate iodide, mp 100°C, lit. 103°C (62).

N,N-Dimethyl-N'-acetylethylenediamine was prepared by treatment of N,N-dimethylethylenediamine with acetic anhydride in toluene and converted to the hydrochloride by HCl in ether, mp 142°C (63). This was converted to 2-acetamidoethyltrimethylammonium iodide by treatment with methyl iodide in ethanol, mp 136-140°C, lit. 139-140°C (63).

(5) Results

A. Fluorimetric Assay (47-49)

Study of labeling by [^{14}C]-BrPin of AcChE isolated from T. nobiliana has been continued. Calculation of the number of ^{14}C introduced per enzyme unit inactivated required an initial determination of enzyme unit content, and from this the value of k_{cat}, which had not been reported previously for AcChE from this source. The value of k_{cat} and rate determinations led to values of enzyme unit content, enzyme unit inactivated and enzyme unit protected by reversible inhibitors, when present. Scintillation counting and the value of specific activity of the 1-[^{14}C]-acetyl chloride then led to values of ^{14}C intro-

duced per unit inactivated, and ^{14}C excluded per enzyme unit protected.

In the previous Final Report (26), the value of k_{cat} from T. californica, 5.5×10^3 sec^{-1} (49), was used in these calculations. At the outset of the current contract a value was calculated from the apparent burst release of thiocholine from reaction with dimethylcarbamylthiocholine (DMCSCh), observed as in the Ellman assay. This was subsequently found to be in error, probably an artifact of the presence of trace thiocholine starting material in the DMCSCh. The assay has now been carried out fluorimetrically by burst release of M7HQ by reaction of the enzyme with the M7HQ dimethylcarbamate (M7CQ) according to literature procedures for assay of AcChE from other sources (47-48), Fig. 1.

In the fluorimetric assay, the burst value is determined by back extrapolation of the slow increase in amplitude after the rapid release, and from this slow increase the rate constant for decarbamylation is calculated, 1.5×10^{-4} sec^{-1}. The rate of carbamylation was determined at 0.475 μM M7CQ, 0.1 of the concentration used in the burst titration, $k_{app} = 3.8 \times 10^{-3}$ sec^{-1}, and the rate constant for carbamylation in the complex is about 0.08 sec^{-1}, Fig. 2. The enzyme content of our purified concentrate is 10 μM, about 65% of the protein content, essentially all of which appears at about 70 kDa on SDS-PAGE. Values of K_m and k_{cat} were determined by hydrolysis of five concentrations of acetylthiocholine (AcSCh), 0.017-0.25 mM, by six concentrations of AcChE, 0.12-1.22 nM: $K_m = 0.056$ mM, as compared with 0.077 mM for enzyme from T. marmorata; $k_{cat} = 4.0 \times 10^3$ sec^{-1}, as compared with 5.5×10^3 sec^{-1} for enzyme from T. californica (49). The value of k_{cat} was determined from the slope of V_{max} vs. enzyme concentration, which is preferable to determination from V_{max} at a single enzyme concentration. The V_{max} vs. [E] data led to a small negative y-intercept, corresponding to apparent loss of activity of 0.05 nM enzyme, which in turn would lead to a low value of k_{cat} if calculated from a single low concentration of enzyme.

Purchased Electrophorus AcChE was examined briefly by the fluorimetric assay, indicating about 16% active enzyme content, $k_{cat} = 1.5 \times 10^4$ sec^{-1}, lit. 1.3×10^4 sec^{-1} (48). It showed decarbamylation rate constant, 4.6×10^{-4} sec^{-1}, lit. 4.2×10^{-4} sec^{-1} (48) and carbamylation $k = 0.10$ sec^{-1}, lit. 0.084 sec^{-1} (48), these values supporting the validity of our assay of enzyme from T. nobiliana.

3. Effects of [^{14}C]-BrPin, Aldrich BrPin, and TAP on Torpedo AcChE at pH 7.0: BrPin K_i = 0.2 mM, TAP K_i = 0.075 mM

(I) Denaturation of affinity purified Torpedo AcChE is much greater at low than at high concentration:

A solution of 0.02 μM AcChE lost activity regularly, 40% in 1.75 hr. In the presence of 0.48 mM [^{14}C]-BrPin there was an increased loss of activity, 30% in 10 min, 75% in 65 min, 86% in 1.75 hr. At 3.6 μM the enzyme showed no spontaneous loss of activity over 2.25 hr, and a regular decrease in activity when incubated with [^{14}C]-BrPin.

(II) Synthesized [^{14}C]-BrPin and purchased BrPin (Aldrich Chemical, Milwaukee, WI) have similar inactivating effects on Torpedo AcChE, and similar protection by TAP:

A solution of about 0.024 μM enzyme alone lost 24% of its activity in 1 hr, and similar amounts, 29% and 22%, in the presence of 1.0 mM [^{14}C]-BrPin and 20 mM TAP, and 1.0 mM BrPin and 20 mM TAP, respectively. In the absence of TAP, 1.0 mM [^{14}C]-BrPin led to 93% inactivation and 1.0 mM BrPin to 72% inactivation also in 1 hr. This particular set of data appears to have greater than the normal error of ±10%, but the solution of ^{14}C-BrPin used was over a month old and we have noted that such data are not always reliable.

Solution of 0.026 μM enzyme in another set of experiment lost 11% activity in 1 hr, 36% in 2 hr, while the solution containing 1 mM freshly prepared ^{14}C-BrPin lost 65% in 1 hr and 92% in 2 hr, and that containing 1 mM commercial BrPin lost 65% in 1 hr and 91% in 2 hr showing freshly prepared ^{14}C-BrPin and commercial BrPin behave similarly toward Torpedo AcChE.

Solutions of 0.012 μM AcChE with 1 mM [^{14}C]-BrPin and 1 mM BrPin lost activity with $t_{1/2}$ values of 21 min and 25 min respectively, about 4 times the spontaneous denaturation rate.

(III) Inactivation of AcChE by [^{14}C]-BrPin is initially prevented by a high concentration of TAP (over 15 mM):

Portions of 1.24 μM AcChE and 1.0 mM in [^{14}C]-BrPin, with varying concentrations of TAP, were assayed after standing 90 min (inactivation of Torpedo AcChE is very slow when the enzyme concentration is greater than 1 μM), showing 56% inactivation in the absence of TAP, 15% inactivation in the presence of the [^{14}C]-BrPin and 3 mM TAP, consistent with concentrations and K_i values of BrPin and TAP, and little, if any, inactivation at 24 and 40 mM TAP. The solutions were put on PAGE and 70 kDa bands were counted: 1.9 ^{14}C introduced per enzyme unit inactivated in the absence of TAP; 1.05 ^{14}C excluded per enzyme

unit protected at 24 and 40 mM TAP; 0.95 ^{14}C excluded per enzyme unit protected at 3 mM TAP. TAP and BrPin both bind reversibly to AcChE and the action of TAP is to decrease the rate of irreversible covalent binding of BrPin residue to AcChE.

(IV) Protection by TAP is reduced during long-term (23-hr) incubation with [^{14}C]-BrPin:

Aliquots of 4 μM Torpedo AcChE was incubated with 1 mM [^{14}C]-BrPin alone and in the presence of 17 mM TAP, leading to 33%, 50% and 90% inactivation after 3.25, 6, and 23 hr in the absence of TAP, 0, 15% and 55% inactivation respectively in the presence of TAP. Aliquots were made 0.1 M in sodium azide after each period and subjected to PAGE, and the 70 kDa bands were counted. At 33% inactivation, 2.15 ^{14}C per enzyme unit inactivated were introduced, 0.95 ^{14}C per enzyme unit protected were excluded in the presence of TAP; at 50% inactivation, 1.5 ^{14}C introduced per enzyme unit inactivated, 0.8 ^{14}C excluded per enzyme unit protected; and at 90% loss of activity, 1.25 ^{14}C introduced per enzyme unit inactivated, 1.0 ^{14}C excluded per enzyme unit protected.

Prolonged incubation led to nearly complete inactivation and to increased introduction of total ^{14}C, but only to about 1.1 ^{14}C per total enzyme unit. However, inactivation despite the presence of TAP may be due to spontaneous denaturation accompanied by non-specific introduction of ^{14}C, in addition to inactivation by [^{14}C]-BrPin by reaction in the trimethyl site. In each case, to the extent that protection by TAP against BrPin inactivation occurs, it prevents introduction of about one ^{14}C per unit protected.

(V) At low concentration of [^{14}C]-BrPin, 0.25 mM, limited inactivation occurs at pH 7.0, and this is not prevented by PTA, but PTA does reduce incorporation of ^{14}C:

Reaction of 1.15 μM Torpedo AcChE with 0.25 mM [^{14}C]-BrPin at pH 7.0 in the presence and absence of 17 mM PTA was examined. At this low concentration of BrPin, there was an initial inactivation, 18-24% over 4.25 hr, beyond a slow denaturation, about 2%/hr, in both the presence and absence of PTA, and then no further inactivation beyond the continuing denaturation. In contrast to the failure of PTA to prevent inactivation, it decreased introduction of ^{14}C by 45% over the first 1.75 hr and by 32% after 4.25 hr incubation. In the absence of PTA, 1.4-1.7 ^{14}C was introduced per enzyme unit inactivated over 1.75 hr, decreased to 0.8-0.9 in the presence of PTA. After 4.25 hr, 2.0 ^{14}C was introduced per enzyme unit inactivated in the absence of PTA, 1.4 ^{14}C in its presence.

In the early stages, PTA appears to be preventing that reaction of [^{14}C]-BrPin which does not lead to inactivation, in

which it presumably alkylates a nucleophile not essential to the enzymic action, possibly at the aryl site. Over the longer period, a slower, more random reaction may occur which is not affected by the active-site-directed inhibitor. Enzyme already labeled by BrPin may no longer bind PTA and additional BrPin may be introduced in time at a position normally protected by the association with PTA such as the aryl site.

(VI) Inefficient protection by PTA led us to examine it in inactivation of Electrophorus AcChE:

Incubation of 17 nM enzyme with 1 mM BrPin for 200 min led to 35% inactivation, completely protected by 1 mM PTA. After incubation for 21 hr, 90% inactivation by BrPin was decreased to 50% by the presence of PTA. Significant protection by PTA of the more stable Electrophorus enzyme is observed. The difference in behavior of Torpedo and Electrophorus AcChE towards protection by PTA is unexpected and the reason is not clear and this merits further study as time permits.

(VII) Since spontaneous denaturation may occur during incubations, particularly in dilute enzyme solutions, introduction of ^{14}C into active and denatured enzyme was compared:

Aliquots of 0.3 μM Torpedo AcChE, (i) fully active and (ii) spontaneously 90% denatured by standing at room temperature for 38 hr, were treated with 1 mM [^{14}C]-BrPin for 2.5 hr. Active enzyme solution lost 50% of its activity and 1.4 ^{14}C per initial enzyme unit (2.9 ^{14}C per enzyme unit inactivated) was incorporated; denatured enzyme had 0.7 ^{14}C per initial enzyme unit. Thus, [^{14}C]-BrPin may react more slowly with denatured enzyme, or not at all, but with nonenzyme-protein present.

Binding of TAP in the active site might protect AcChE against spontaneous denaturation:

Effects of TAP on denaturation and on inactivation by BrPin of Torpedo AcChE were examined in solutions of (i) 0.3 μM AcChE, (ii) 0.3 μM AcChE and 18 mM TAP, and (iii) 0.3 μM AcChE, 18 mM TAP and 0.9 mM BrPin. Solutions (i), (ii), and (iii) lost no activity in 2 hr; (i) and (ii) lost none in 24 hr, (iii) lost 28%; (i) lost 90% of its activity after 72 hr, (ii) about 10% and (iii) 60%. TAP appeared to protect AcChE against denaturation, but this had not been observed in an earlier similar experiment. TAP gave partial protection during long incubation with BrPin.

TAP might not protect denatured enzyme against incorporation of [^{14}C]-BrPin because efficient binding of TAP requires strong interaction of its carbonyl at the esteratic site, in addition to the quaternary substituent binding in the trimethyl site. For information about this, protection by TAP against

[^{14}C]-BrPin was examined in DFP-inactivated enzyme, in which no further interaction at the esteratic site may occur.

Aliquots, 0.64 mL of 1.7 μM (i) active and (ii) DFP-inactivated enzyme were incubated for 2.5 hr with 0.6 mM [^{14}C]-BrPin (a) in the absence and (b) presence of 22 mM TAP, leading to 21% inactivation in (ia) and none in (ib). 5.1×10^{-10} moles ^{14}C were incorporated (2.1 ^{14}C introduced per enzyme unit inactivated) in the absence of TAP and 3.1×10^{-10} moles ^{14}C in the presence of TAP (0.8 ^{14}C excluded per enzyme unit protected). In the DFP-inactivated enzyme, the corresponding figures were 4.0×10^{-10} moles in the absence of TAP and 3.2×10^{-10} mole ^{14}C, respectively. Thus, [^{14}C]-BrPin appeared to enter the trimethyl site of DFP-inactivated enzyme more slowly than in active enzyme, and this part was prevented by TAP, the same ^{14}C content being found in both enzymes in the presence of TAP. This may indicate that the excess over one ^{14}C introduced in inactivation by [^{14}C]-BrPin is not entering the trimethyl site of enzyme merely inactivated or denatured by loss of an effective esteratic site.

C. Inactivation of Torpedo AcChE and Effects of TAP, PTA and DMMP at pH <7

Inactivation by [^{14}C]-BrPin is more rapid at pH 6 than at pH 7, denaturation may not increase excessively and protection by TAP is observed. PTA does not appear to protect against inactivation, but PTA, like TAP, decreases incorporation of ^{14}C. Similarly, 3-dimethylamino-N-methylpyridinium ion (DMMP) offered little protection against inactivation, while reducing incorporation of ^{14}C. More rapid inactivation of enzyme at low pH is helpful at particularly high concentrations (>1 μM) of enzyme needed for isolation of labeled peptide fragments for identifying the amino acid residue(s) labeled by ^{14}C-BrPin.

(I) Inactivation of 0.4 μM AcChE by 0.4 mM [^{14}C]-BrPin at pH 6.0 was examined alone and in the presence of 14 mM TAP and 12 mM PTA. After incubation for 90 min there was 9% denaturation in the absence of additives, 57% inactivation by the BrPin, complete protection by TAP, no protection in the presence of PTA; 1.9 ^{14}C was introduced per enzyme unit inactivated, and 0.9 ^{14}C was excluded per unit protected by TAP. PTA, while not protecting against inactivation, decreased ^{14}C incorporation by 35%.

(±I) Inactivation of 0.45 μM AcChE by 0.1 mM [^{14}C]-BrPin at pH 6.0 was examined in the presence of 2.7 mM DMMP. After 2.5 hr, enzyme alone lost no activity within the experimental error, ±10%; enzyme plus DMMP was stable; enzyme plus BrPin lost 80% activity; and enzyme, BrPin and DMMP lost 60%. The pyridinium compound, even with a dimethylamino substituent, did

not provide effective protection against inactivation by [^{14}C]-BrPin; it reduced introduction of ^{14}C by 40%.

(III) At slightly lower pH, 5.7, incubation of 0.6 μM AcChE with lower concentration of [^{14}C]-BrPin, 0.15 mM, for 50 min, alone and in the presence of 14 mM TAP, led to 53% inactivation by [^{14}C]-BrPin, reduced to 15% in the presence of TAP, as compared with 19% denaturation in the absence of additives. Less ^{14}C was introduced as the inactivation rate increased at lower pH, 1.4 ^{14}C introduced per enzyme unit inactivated, and 0.9 ^{14}C excluded per enzyme unit protected in the presence of TAP.

(IV) At pH 5.4 inactivation by [^{14}C]-BrPin is very rapid and denaturation is also increased substantially. In an initial experiment, low incorporation of ^{14}C at low enzyme and low [^{14}C]-BrPin concentrations indicated excessive denaturation. Incubation of 0.29 μM AcChE and 0.15 mM [^{14}C]-BrPin led to complete inactivation in less than 25 min and 58% inactivation in the presence of 18 mM TAP: 0.35 ^{14}C introduced per enzyme unit inactivated in the absence of TAP and 0.32 ^{14}C excluded per enzyme unit protected by TAP.

(V) At pH 5.4, at slightly higher concentrations of [^{14}C]-BrPin and enzyme, rapid inactivation, satisfactory protection and ^{14}C incorporation and exclusion were observed. Incubation of 0.4 μM AcChE with 0.4 mM [^{14}C]-BrPin, alone and in the presence of 14 mM PTA and 15 mM TAP, for 10 min, after which azide was added, led to 94%, 90% and 40% inactivation with no denaturation in a control. Introduction of ^{14}C was 0.8 ^{14}C per enzyme unit inactivated by [^{14}C]-BrPin alone; 0.7 ^{14}C excluded per enzyme unit protected. PTA, while offering minimal protection, decreased ^{14}C introduction by 38%, although there was no excess, and if anything a deficiency, of ^{14}C for inactivation.

Incubation of 0.65 μM AcChE and 0.8 mM [^{14}C]-BrPin at pH 5.4 led to 91% inactivation in 23 min and to 55% inactivation in the presence of 19 mM TAP; 1.0 ^{14}C introduced per enzyme unit inactivated, perhaps 1.1 ^{14}C introduced per enzyme unit when corrected for denaturation during the short period of inactivation, and 1.4 ^{14}C excluded per enzyme unit protected by TAP.

We have previously reported (26) possible acid catalysis of inactivation of Torpedo AcChE by BrPin. This effect has now been found not to occur in inactivation of purified Electrophorus AcChE by BrPin or by phenacyl bromide.

D. Inactivation at pH 6

Inactivation at pH 6 appeared to provide satisfactorily rapid reaction of BrPin combined with enzyme stability. A series of studies was carried out with Torpedo AcChE.

(I) Incubation of 0.66 μM AcChE with a 0.3 mM combination of equal parts of [^{14}C]-BrPin and "cold" BrPin for 85 min led to 80% inactivation, with none in a control and in the presence of the BrPin and 15 mM TAP. TAP decreased ^{14}C incorporation by 43%.

(II) Incubation of 0.74 μM AcChE with 0.2 mM [^{14}C]-BrPin for 70 min led to 54% inactivation, completely protected by a combination of 9 mM TAP and 10 mM 3-tert-butylphenol, with a 64% decrease of ^{14}C incorporation.

(III) Incubation of 0.5 μM AcChE with 0.3 mM [^{14}C]-BrPin, alone and in the presence of 13 mM TAP plus 13 mM PTA for 105 min, led to 80% inactivation and complete protection, respectively, and a 51% decrease in ^{14}C incorporation by the reversible inhibitors.

Incubation for 105 min of 0.66 μM AcChE with 0.17 mM [^{14}C]-BrPin alone led to 70% inactivation, and, in the presence of 14 mM TAP and of 0.25 mM 3-trimethylammonioacetophenone (TMAAPh), $K_i = 5 \times 10^{-4}$ mM (64), to protection by each of these additives. Inactivation incorporated 1.6 ^{14}C per enzyme unit inactivated, protection excluded 1.1 ^{14}C per enzyme unit protected. TMAAPh is the first benzene derivative to protect as effectively as TAP.

E. Decamethonium (DEC)

DEC was examined as a protecting agent for Torpedo AcChE and compared with TAP and PTA. Incubation at pH 7.0 for 160 min of 0.50 μM AcChE with 0.5 mM [^{14}C]-BrPin led to 37% inactivation, completely prevented by 16 mM TAP or 3.5 mM DEC. Similar incubation with TAP at pH 6.0 for 75 min led to 76% inactivation by [^{14}C]-BrPin and effective protection by TAP. DEC reduced ^{14}C incorporation by 45% at pH 7.0; TAP excluded 60% ^{14}C at pH 6.0.

Incubation of 16 nM AcChE for 40 min at pH 6.0 led to 33% inactivation in a control; 0.5 mM "cold" BrPin led to 89% inactivation, reduced to 50% by 7.0 mM PTA, and 40% by 0.3 mM DEC. Dilute purified Torpedo AcChE is unstable at pH 6.0, is rapidly inactivated by BrPin, is protected by PTA to some extent, and is protected somewhat more effectively by DEC.

F. Gel Filtration of Tryptic Digest of Labeled Torpedo AcChE

(I) In a preliminary isolation, Torpedo enzyme concentrate, 2.0 mL, containing 2.5×10^{-8} moles of active enzyme, was diluted to 3.5 mL with pH 7 phosphate buffer, made 0.15 mM in

[^{14}C]-BrPin, leading to 49% inactivation in 120 min, and treated with 0.32 mL of 1.0 M sodium azide to stop inactivation. This was incubated with dithiothreitol, treated with iodoacetic acid and then with dithiothreitol again, dialyzed, subjected to tryptic cleavage (65) and to G-50 Sephadex gel filtration; 3-mL fractions were collected and examined at 219 nm and by scintillation counting. The fractions containing ^{14}C were pooled, 35 mL, dried, resuspended in 3 mL of 50 mM NH_4OH and applied again to the G-50 Sephadex column along with bradykinin, M_r 1240; 3-mL fractions were again collected and examined at 219 nm and by scintillation counting. The fractions containing ^{14}C appear to be of M_r about 1300. These were later rechromatographed, pooled, dried, resuspended in 0.2 mL of distilled water containing 0.1% TFA and analyzed by HPLC, 20 μL aliquots, C-18 Dynamax 300Å column, linear gradient from H_2O:0.1% TFA to 50% CH_3CN:0.05% TFA, MacIntosh HPLC program, 0.2 mL fractions, Gilson-203 programmable collector, analyzed at 219 nm and for ^{14}C in 10 mL ACS scintillation fluid. Corrections for 0.4-0.5 mL void between spectrophotometer and fraction collector were applied. Two ^{14}C peaks were observed, one with a shoulder near but apparently not superposed on 219 nm absorption peaks.

(II) Similar experiments are now in progress in which 8.5 mL aliquots of 2 μM AcChE were incubated for 150 min with (i) 0.15 mM [^{14}C]-BrPin or (ii) with 0.075 mM [^{14}C]-BrPin plus 0.075 mM "cold" BrPin, alone and in the presence of 13 mM TAP at pH 6.0; 40% inactivation was observed in (i), 50% in (ii) with incorporated ^{14}C equal to half that in (i). The incubates with TAP and a control without additives showed no inactivation. The five samples are being chromatographed.

G. Pyridine Derivatives as Inhibitors and Protecting Agents

Evaluation of pyridine derivatives as reversible inhibitors and as possible irreversible inactivators and potential labeling agents was continued in titrimetric studies on Electrophorus AcChE. Results on reversible inhibition obtained previously (26) and in the present study are combined and summarized in Table III: Pyridine has $K_{i(com)}$ = 8.1 mM at pH 7.8, as compared with 12.1 mM at pH 8.5 (26); 4-tert-butylpyridine has $K_{i(com)}$ = 1.3 mM at pH 7.8 as compared with 3.7 mM at pH 8.5 (26). Additional compounds were examined at pH 7.8: 2-methoxypyridine, $K_{i(com)}$ = 4.0 mM; 3-bromoacetylpyridine hydrobromide, as a reversible inhibitor, has $K_{i(com)}$ = 2.1 mM, $K_{i(nonc)}$ = 1.8 mM, similar to 3-acetylpyridine; 4-nitropyridine-N-oxide has $K_{i(com)}$ = 2.1 mM, $K_{i(nonc)}$ = 2.7 mM, as compared with pyridine-N-oxide, $K_{i(com)}$ = 11 mM, $K_{i(nonc)}$ = 63 mM (26); 4-nitropyridine has $K_{i(com)}$ = 2.0 mM, $K_{i(nonc)}$ = 2.8 mM, similar to its N-oxide.

N-Methylpyridinium iodide (NMP) had $K_{i(com)}$ = 0.16 mM, $K_{i(nonc)}$ = 0.2 mM at pH 7.8, reported 0.11 mM at pH 7.0 (66). NMP was examined as a protecting agent against inactivation of Torpedo AcChE by BrPin. Results are inconsistent: With dilute (21 nM) enzyme, spontaneous denaturation at pH 7.0 was rapid, 52% in 130 min, apparently decreased to 33% by 43 mM NMP; 95% inactivation occurred in the presence of 1.0 mM BrPin in this period, decreased to 56% by 43 mM NMP. However, at high enzyme concentration, 0.33 μM, at which spontaneous denaturation did not occur, 43 mM NMP appeared to cause some inactivation, 30% in 45 min, and did not decrease inactivation by 2.5 mM [^{14}C]-BrPin, 70% in the same period. Weak protection by DMMP was described in section C(II).

These inconsistent results, obtained with a [^{14}C]-BrPin solution several months old, led us to consider the possible instability of such solutions in moist ether, which turned yellow while stored in a freezer. For example, the Br_2Pin present might lead to tert-butylglyoxal. An attempt to prepare this from BrPin and dimethylsulfoxide led to a product which seemed to polymerize rapidly. The glyoxal group was examined briefly with phenylglyoxal. This is an effective, purely competitive reversible inhibitor for Torpedo AcChE, K_i = 0.56 mM. It led to some inactivation, about twice that of the denaturation of dilute Torpedo enzyme. Further examination indicated that [^{14}C]-BrPin solution is stable over the time scale of our intended use.

In brief examination of pyridine derivatives as irreversible inactivators, 3-bromoacetylpyridine proved effective, 3 mM inactivating Electrophorus AcChE at pH 7.8 with $t_{1/2}$ = 1.8 hr, as compared with 4 hr for the benzene analogue, phenacyl bromide. However, 2-oxyrilpyridine, $K_{i(com)}$ = 1.5 mM, Table III, at 3 mM in 23 hr incubation at pH 7.8 did not inactivate, while styrene oxide did (26). Also, 3-chloromethylpyridine, 3 mM, did not inactivate AcChE.

H. Noncompetitive Inhibition of Hydrolysis by AcChE

Results of a study of six pairs of tetrasubstituted and trisubstituted inhibitors and five substrates are summarized in Table IV. Some kinetic constant values are updated and are not identical to the values reported in earlier studies (Table I), but the changes do not affect the earlier conclusion (Sect. 2 A I). The inhibitors are tetra- and trimethylammonium ions, IA and IB, choline and its lower homologue, IIA and IIB, choline ethyl ether and its lower homologue, IIIA and IIIB, TAP and its lower homologue, IVA and IVB, 2-acetamidoethyltrimethylammonium iodide ("acetylazacholine") and its lower homologue, N,N-dimethyl-N'-acetylethylenediamine, VA and VB, and tert-butyl- and isopropylammonium ions, VIA and VIB. The substrates are acetylcholine, studied with all the inhibitors,

a less reactive, cationic, higher homologue, 2-N,N-diethyl-N-butylammonioethyl acetate (EBAAc), studied with five inhibitor pairs, the uncharged carbon analogue of AcCh, 3,3-dimethylbutyl acetate (DMBAc) studied with all the inhibitors, methylsulfonylethyl acetate (MSEAc), a sulfonyl analogue of DMBAc, studied with three inhibitor pairs, and n-butyl acetate (BAc) studied with five inhibitor pairs.

The competitive binding constants in five pairs, corresponding to binding to free enzyme, are more favorable (i.e., lower $K_{i(com)}$) for the tetra-substituted than for the trisubstituted analogues, whether the N^+ cation is completely shielded as in the quaternary ammonium compounds, or fully exposed, in the tert-butyl and isopropyl amines. Four alkyl substituents favor binding to the free enzyme over three. In the case of the amides, VA and VB, the competitive bindings of the two are similar. Most favorable binding of the tetra- and tri-substituted compounds is found in the ketones, IVA and IVB, 0.03 and 1 mM, respectively, probably arising from interaction of the carbonyl groups with serine hydroxyl. tert-Butyl- and isopropylammonium ions, VIA and VIB, bind more strongly than their isomers, IA and IB, indicating a favorable additional effect of a fully exposed $-NH_3^+$ group in both four and three carbon systems. Hydroxyethyl and ethoxyethyl groups in pairs II and III improve binding over methyl, interacting in a lipophilic domain between the trimethyl and esteratic sites, probably related to the aryl binding site.

Tetrasubstituted compounds, IA, IIA, IIIA, VA and VIA, show less favorable noncompetitive binding, higher $K_{i(nonc)}$, with the most rapid substrate, AcCh, and more favorable noncompetitive binding with the less reactive substrates. $K_{i(nonc)}$ values generally decrease with decreasing enzymic reactivity and they become comparable to the effective competitive binding values. They show this with both cationic EBAAc and uncharged DMBAc, MSEAc and BAc. In no case is $K_{i(nonc)}$ more favorable for AcCh. Hydrolysis by AcChE (E) proceeds via formation of the enzyme-substrate complex (ES), acetylation of the enzyme, k_2, forming the acetylenzyme (EA), and hydrolysis of EA to regenerate E, k_3:

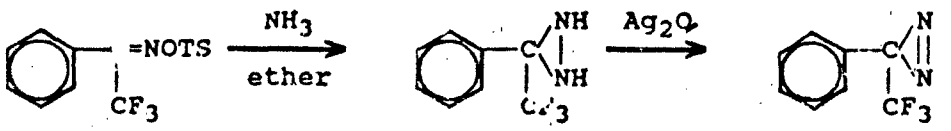
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E$$

The value of k_3 is the same for all acetate ester substrates. Highly reactive substrates have high k_2 values, low ES and high EA concentrations in the steady state. We conclude that noncompetitive inhibition by tetrasubstituted compounds involves binding, or preferential binding, with ES, forming ESI, rather than with EA. The most effective inhibitor, IVA, shows no noncompetitive component.

Trisubstituted compounds generally show more favorable noncompetitive binding with AcCh than with the less reactive substrates. This is found with IB except for DMBAc, and with IIB, IIIB, IVB, but not with VB and VIB. $K_{i(nonc)}$ values for trisubstituted inhibitors with AcCh are no more favorable than $K_{i(nonc)}$ values for tetrasubstituted inhibitors with the less reactive substrates. By the currently accepted logic, we conclude that noncompetitive inhibition by trisubstituted compounds generally involves preferential binding to EA, forming EAI. The amide VB and the primary ammonio compounds, on the other hand, appear to bind preferentially to ES, forming ESI. The uncharged analogue of AcCh, DMBAc, seems to show the greatest tendency to strong noncompetitive inhibition by trisubstituted compounds, comparable to that of AcCh. With the highest value of k_{cat} among the slow substrates, one-fourth that of AcCh, and K_s three times that of AcCh, binding to EA may be favored.

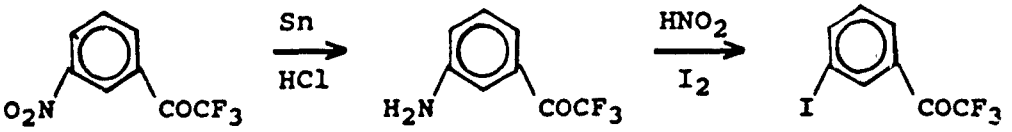
I. Preparation of 3-Phenyl-3-trifluoromethyldiazirine (PTDZ) and 3-(3'-Iodo)phenyl-3-trifluoromethyldiazirine (IPTDZ)

Trifluoroacetophenone was converted to its oxime and oxime tosylate, and treated with liquid NH_3 in ether at room temperature in a sealed tube overnight, leading to the diaziridine. This was oxidized to the diazirine by silver oxide in ether (67-68). Oxidation of the diaziridine to the diazirine with silver oxide did not proceed well when the Ag_2O was prepared as described in the synthesis of these materials. The procedure in Organic Syntheses 5, 897 (69) led to effective Ag_2O. A solution of 350 mg of diaziridine in ether was stirred under nitrogen with Ag_2O prepared from 0.8 g of silver nitrate for 5 hr, leading to PTDZ in 40% yield, determined from $\epsilon_{353} = 266$ in ethanol. The diaziridine precursor was prepared in 88% yield by treatment of the ketoxime tosylate with liquid ammonia in ether in a sealed tube at room temperature for 24 hr.

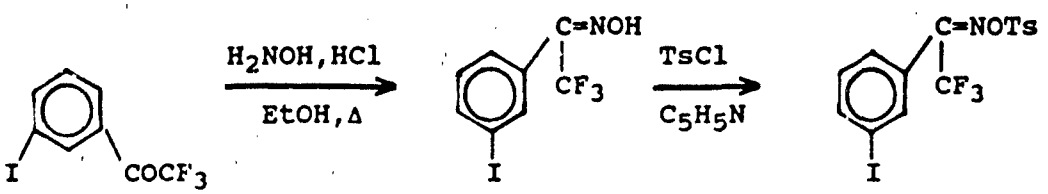


The iodo derivative, IPTDZ, was prepared similarly. Trifluoroacetophenone was nitrated (70) and the meta-nitro derivative was reduced with tin in HCl (71), leading to a red oil with suspended solid. This was purified by chromatography on silica, leading to 42% yield of the meta-amino compound, with appropriate NMR. A red by-product was retained in the column, and later fractions appeared to contain some of the

carbinol reduction product. The amino compound was diazotized



with sulfuric acid and sodium nitrite, and treated with potassium iodide, forming m-iodotrifluoroacetophenone in 60% yield. This showed one spot on TLC but the proton NMR in the aromatic region was complex, possibly indicating the presence of a phenolic product. A portion, 3 g, was converted to the oxime, an oil, 2.5 g, 80%, which showed two spots in TLC,



possibly cis and trans oximes. This was converted to the tosyloxime, 2.8 g, 75% yield of crude product, which was purified on silica and converted to the diaziridine in 90% yield, and this was oxidized to the diazirine in 35% yield.

J. Effects of 5-Trimethylammonio-2-pentanone and Diisopropyl Fluorophosphate on Reactions of [^{14}C]-BrPin with AcChE from T. nobiliana (72)

A manuscript was prepared describing the synthesis of [^{14}C]-BrPin from 1-[^{14}C]-acetyl chloride and tert-butylmagnesium chloride with cuprous chloride catalyst; the isolation of AcChE from T. nobiliana and its fluorimetric assay by burst release of N-methyl-7-hydroxyquinolinium ion from the N,N-dimethylcarbamate; determination of its K_m = 0.055 mM and k_{cat} = 4.0 x 10^3 sec^{-1} in hydrolysis of acetylthiocholine; its reaction with BrPin, first reversibly, K_i = 0.20 mM, then with irreversible inactivation; introduction of 1.9 ^{14}C per enzyme unit inactivated at 50% inactivation at pH 7.0; protection against this inactivation by 5-trimethylammonio-2-pentanone, K_i = 0.075 mM, and prevention of introduction of 1.0 ^{14}C per unit of enzyme protected; prevention of introduction of [^{3}H]-diisopropyl fluorophosphate by prior inactivation by BrPin; introduction of ^{14}C from [^{14}C]-BrPin after prior inactivation by DFP, and retention of both BrPin and DFP in the enzyme. This failure of [^{3}H]-DFP to enter AcChE preinactivated by BrPin might have been due to reversible inhibition by the continued presence of BrPin solution. However, treatment of Torpedo

AcChE by 9 μM DFP in the presence and absence of 0.9 mM BrPin led to complete inactivation in 20 min. This indicated no significant retardation of introduction of [^{3}H]-DFP by BrPin in solution. Therefore it is covelently bound BrPin which prevents reaction of DFP with the enzyme, just as it prevents reaction of substrates. However, BrPin does not react with the esterolytic serine hydroxide, since prior rection there with DFP does not prevent subsequent introduction of [^{14}C]-BrPin.

K. Studies with [^{3}H]-Soman

At the end of this reporting period analogous experiments were initiated with soman and [^{3}H]-soman in collaboration with Dr. C. Broomfield, Biochemical Pharmacology Branch, USAMRICD, APG-EA. AcChE samples from T. nobiliana were (i) inactivated by BrPin at Brandeis and taken by Dr. Broomfield to Aberdeen Proving Ground, MD for treatment with [^{3}H]-soman; (ii) inactivated at Aberdeen Proving Ground, MD by soman and [^{3}H]-soman and sent to Brandeis for treatment with [^{14}C]-BrPin. Preinactivation by BrPin prevented subsequent introduction of ^{3}H from [^{3}H]-soman, and preinactivation by soman retards subsequent introduction of ^{14}C from [^{14}C]-BrPin. This work is in progress and details will be presented in a later report.

(6) Discussion and Conclusions

We have determined the enzyme content and kinetic constants of purified AcChE from T. nobiliana. The kinetic constants are similar to but not identical with those for AcChE from other related sources. This allows determination of the number of enzyme sites and thus the number of ^{14}C atoms introduced in inactivation by [^{14}C]-BrPin: We find this number to be 2. Prevention of this inactivation by the presence of TAP, the ketone analogue of the natural substrate, AcCh, supports the view that the reaction of BrPin occurs in the active site, in the trimethyl subsite, and that identification of the labeled amino-acid will be informative as to the composition of the active site.

The prevention by TAP of this inactivation excludes one ^{14}C per unit protected, and analysis of such protected, but partly labeled, enzyme will assist in determination of the essential protected amino acid, and the other labeled amino acid(s). Unlabeled, [^{14}C]-BrPin-inactivated, and TAP-protected enzyme samples have now been subjected to tryptic digestion, the peptides are being isolated, and amino acid analyses will soon be carried out. For this purpose inactivation is carried out at pH 6, at which it is much more rapid than at higher pH.

The BrPin inactivation, by definition, prevents action of substrates at the active site. Similarly, it prevents action

there of the powerful inactivating agents DFP and soman, which are pseudosubstrates, utilizing the esteratic mechanism to acylate the essential serine. This is demonstrated by prevention by prior BrPin inactivation of introduction of ^{3}H from [^{3}H]-DFP and [^{3}H]-soman. That prior inactivation by DFP does not exclude ^{14}C from [^{14}C]-BrPin and that this subsequent reaction with BrPin does not displace the [^{3}H]-DFP indicate that occupation of the esteratic site by moieties of DFP does not prevent occupation and reaction at the trimethyl site by BrPin. Soman studies will be continued.

Reversible inhibition by derivatives of benzene, strengthened by electron-attracting substituents and by dimethylamino substituents, and inhibition by derivatives of phenol, which is greatly strengthened by meta-tert-butyl and isopropyl substituents, led us to propose binding of aromatic compounds via charge-transfer interaction with aromatic amino acid sidechains at an aryl site, contiguous to the trimethyl site, but not occluding it, and by dispersion force interaction of the hydrocarbon substituents in the trimethyl site (38). Binding of the aryl group itself would interfere with binding of the $-CH_2OCOCH_3$ group of substrates at and near the esteratic site, leading to inhibition. However, nitrobenzene and acetophenone, like small cations (39), occupying part of the active site, accelerate hydrolysis of ethyl acetate, which has no group occupying the trimethyl site. This effect is not due to positive charge. The second species present, cationic or uncharged, may firm up the active site, improving the fit of the small substrate with the functional groups of the esteratic site.

Consistent with this, nitrobenzene, albeit at low concentration, not occluding the trimethyl cavity, did not prevent inactivation by [^{14}C]-BrPin, nor did NMP. NMP did reduce ^{14}C incorporation. Apparently the trimethyl and aryl sites may be occupied simultaneously by appropriate compounds. These small aromatic compounds may prevent labeling of a residue not essential to the catalysis, while TAP protects an essential residue. However, DMMP also failed to prevent inactivation, while reducing ^{14}C incorporation, and even PTA gave inconsistent results. The trimethyl site may accommodate larger volumes than tert-butyl or trimethylammonio, as indicated by fairly rapid hydrolysis of 2-N,N-diethyl-N-butylammonioethyl acetate, and might admit and bind both tert-butyl of BrPin at an essential locus, and di- and trimethylammonio substituents of pyridine and benzene at a residue in the trimethyl cavity not essential for the catalysis, but contributing to binding. TMAAPh, which may occlude efficiently the entire space required by AcCh or TAP, and more, prevented inactivation by [^{14}C]-BrPin. This problem may be examined further with reversible inhibitors, which may direct large groups to the trimethyl site. Study of the anticholinesterase agent ketamine is such a possibility. The relation between the trimethyl and aryl sites

will be examined by study of effects of irreversible inhibition by aromatic compounds, such as styrene oxide, phenacyl bromide, and bromoacetylpyridine, on subsequent reaction with [^{14}C]-BrPin, and by study of effects of varied tri- and tetramethyl ammonio reversible inhibitors on inactivation by these aromatic compounds.

Pyridines, Table III, provide potentially uncharged molecules with higher water solubility than the benzenes for study of the aryl site. Their binding is greater at low than at high pH, reflecting protonation of the pyridine despite increased protonation of enzyme carboxylate. Electron-attracting substituents, $-NO_2$ and $-COCH_3$, increase binding, but not as much as in the benzenes, the pyridine nucleus itself being electronegative. This factor may account for pyridine itself binding more strongly than benzene despite its water miscibility. Conversion of pyridines to N-oxides, of uncharged N to dipolar $-N^+-O^-$, has no effect on binding, while in aliphatic systems, conversion of cationic $--NH^+$ to dipolar $--N^+-O^-$, with no net charge, greatly diminishes or eliminates binding.

Hydrocarbon substituents improve binding of pyridines, possibly interacting at the trimethyl site. Amino groups improve binding, donating electrons to the nucleus, increasing basicity of the aromatic N and its degree of protonation. These effects are combined and most marked in the dimethylaminopyridine, leading to high pK_a values, high degree of protonation and strong binding, supporting the contiguous location of the aryl and trimethyl sites. 2-Dimethylaminopyridine (DMAP), with conjugated aliphatic and aromatic nitrogens, binds as strongly as the permanently cationic N-methylpyridinium ions (NMP's), and the binding is only slightly increased in 3-dimethylamino-N-methylpyridinium. Since DMAP may exist in neutral and cationic forms, depending on environment, it provides the possibility of lipid permeability and strong polar, aryl and trimethyl site interaction and binding. Thus, dimethylaminopyridine aldoximes may lead to reactivating dephosphorylation superior to that from N-methylpyridinium aldoximes.

Strong binding of the dimethylaminopyridines supports the contiguous location of the aryl and trimethyl sites. Weak or no protection against [^{14}C]-BrPin by NMP indicates, as with nitrobenzene, that the aryl and trimethyl sites may be occupied simultaneously by two species. Weak protection by 3-DMAP, as in the case of PTA, indicates the need for further study of the size of the trimethyl site, discussed above.

It is surprising that the epoxide 2-oxyrilpyridine did not inactivate AcChE, while the phenyl analogue styrene oxide did. However, bromoacetylpyridine is more effective than its phenyl analogue. Effects of reversible inhibitors related to AcCh, ranging from trimethylammonium ion to TAP, on this inactivation

will be studied. Use of phenacyl bromide, bromoacetylpyridine and styrene oxide as possible labeling agents will be explored. Effects of these aromatic inactivators on exclusion of [^{14}C]-BrPin will also be examined.

Study of noncompetitive inhibition by tetra- and tri-substituted cationic inhibitors of cationic and uncharged substrates of varying reactivity leads to a further criticism of the overly simple view of the anionic site. An anionic site became, it seems, viewed at times as the one anionic site. This encouraged the view that noncompetitive inhibition would not involve binding of cationic inhibitors to the enzyme-substrate complex, ES, since the anionic site in ES is occupied by S, and it therefore would involve binding to the acetylenzyme, EA, in which the anionic site is vacant, the choline moiety having been ejected. This was supported, confirmed it seemed, by more effective noncompetitive inhibition in hydrolysis of most reactive substrates in which highest concentration of EA is present (28). The study was carried out largely with trisubstituted inhibitors, and the interpretation was extended to inhibition by high concentration of substrate itself, AcCh, a tetrasubstituted, quaternary, ammonium ion (28,29,30,64) and to other tetraalkyl ammonium ions. However, calculations based on this mechanism showed purported binding energies to acetylenzyme rising from methyl to trimethylammonium ions, then decreasing with tetramethylammonium ion (30). This decrease was attributed to an unfavorable steric effect. However, higher tetraalkylammonium ions bound more strongly to EA than trimethylammonium ion itself, and would presumably have still less favorable steric properties if that was indeed the relevant effect. Our study shows that the apparent anomaly arises from the mode of binding of the tetraalkylammonium ions being different from that of the lower homologues. The tetrasubstituted analogues show stronger noncompetitive inhibition with the slow substrates, as strong as that of the trisubstituted with the fast substrate, in the 1 mM range, and as strong as the competitive binding. The accepted logic requires that they inhibit preferentially, if not solely, by binding with ES, forming ESI, and that inhibition by substrate AcCh may occur via binding to ES, forming ESS. TAP behaves purely competitively under our conditions, $K_{i(com)}$ = 0.02 mM. If noncompetitive binding is found only in the 1 mM range, a competitive inhibitor as strong as TAP would not and could not be studied at a high enough concentration for the noncompetitive mode to be observed.

The isoelectric point of AcChE is 5.4 (20), leading to excess negative charge at physiologic pH and the pH values of our studies. Effects of ionic strength in binding of alkyl cations are interpreted as indicating presence of 6 to 9 anionic charges in the area about the active site. However, the stronger binding of monocations as compared with structurally related, uncharged inhibitors and substrates is less than

would arise from close contact of anion and cation. There is a glutamic residue adjacent to the active serine (73), but there is no compelling basis for concluding that an anionic charge is located on the surface of the cavity in contact with tert-butyl or trimethylammonio substituents. While this seems to have been the common assumption and depiction, location of the multiple anionic charges is now indicated less precisely (74).

An inorganic ion, Ca^{+2}, does bind, presumably at one or two carboxylates, but this does not interfere with binding of AcCh, and it increases its rate of hydrolysis (75). Decamethonium, an aliphatic quaternary dication, competes with the binding of AcCh, one end probably binding at the trimethyl site, and also competes with the binding of Ca^{+2} (75), its hydrocarbon chain possibly covering a carboxylate. Gallamine, an aryl-centered, aliphatic quaternary trication, does not interfere with the binding of AcCh (76), Ca^{++}, or tetramethylammonium ion, but does interfere with decamethonium, and with inhibition by substrate, AcCh (75).

The highly negatively charged surface of the enzyme accounts for the effects attributed to the "anionic site," and these effects are small indeed, if not entirely absent in the hydrolysis of substrates (12,13,19,25). The binding cavity for the substrate "head" is best described as trimethyl, and the elegantly simple but oversimplified view of noncompetitive inhibition arising from binding to EA is untenable for the compounds more closely related to the natural substrate, i.e., the tetrasubstituted ones. The effects of gallamine, described above, may indicate that the second binding site in ESI and ESS is also that for decamethonium. However, this too may be an oversimplification. Study of an uncharged bifunctional analogue of decamethonium may indicate whether this second site is anionic or also trimethyl in character.

As to photoaffinity labeling, we have prepared two diazirines, and have thought of some aromatic ketones, with which we have considerable experience (77), but have not had the opportunity to pursue this actively yet.

As to literature publications, we have submitted one manuscript recently, will write one on noncompetitive inhibition, and hope to have results soon on the site of labeling by [^{14}C]-BrPin. We have substantial results on the relations of interactions at the aryl and trimethyl subsites and intend to study this further before attempting publication. Our views here, too, will differ from the conventional ones. These studies may be quite informative as to the enzyme surface with which the natural substrate is in contact as it is hydrolyzed.

Table I[a]

Hydrolysis of $X-CH_2CH_2OCOCH_3$ by *Electrophorus* AcChE, in 0.18 M NaCl, 25°C; Molar Refractivity (MR), Hydrophobicity (Π), and Kinetic Constants

Cpd	β-Substituent				$10^9 \times [E]$	$10^{-2} \times k_{cat}$	K_m	$10^{-4} \times (k_{cat}/K_m)$	$k_{(OH^-)}$	$10^{-4} \times (k_{cat}/K_m)_n$
No.	X-	MR (cm^3)	Π	Conc (mM)	M	sec^{-1}	(mM)	$M^{-1}sec^{-1}$	$M^{-1}sec^{-1}$	$M^{-1}sec^{-1}$
I	$(CH_3)_2S^+$-	16.4	-0.50	0.2-1.2	0.6	77	0.33	2300	3.1	2100
IA	$(CH_3)_3N^+$-	17.2	-1.24	0.1-0.6	0.1	160	0.33	4800	2.8	----
II	$(CH_3)_3Si$-	25.0	+2.59	0.1-3.0	0.4	57	3.5	160	0.06	7600
IIA	$(CH_3)_3C$-	19.6	+1.98	0.7-5.3	0.2	66	5.3	125	0.07	5000
III	$(CH_3)_2N^+(O^-)$-	15.0	-3.0	1.5-9.0	0.3	45	18	25	0.82	86
IIIA	$(CH_3)_2CH$-	15.0	+1.53	1.0-5.0	1.1	34	3.6	93	0.11	2400
IV	$CH_3S(O_2)$-	13.9	-1.9	6-21	0.6	11	6.2	18	1.4	36
IVA	$CH_3S(O)$-	14.1	-1.85	7-22	0.5	38	16	24	0.45	150
IVB	O_2N-	6.7	-0.85	7-22	1.1	--	---	(5)	----	----
V	CH_3S-	13.3	+0.45	3-21	0.6	35	15	23	0.28	230
VA	CH_3CH_2-	10.3	+1.02	10-17	0.2	36	13	26	0.11	660
VI	Cl-	5.93	+0.39	4-40	0.2	34	14	25	0.31	220
VIA	Br-	8.80	+0.60	1-12	0.1	45	7.5	59	0.42	400

[a] From Ref. 13. See Ref. 13 for details and references.

Table II[a]

Reversible Inhibition of Hydrolysis of AcCh by Electrophorus AcChE at pH 7.8, 25°C, 0.18 M NaCl

No.	Compd	$K_{I(com)}$, mM	$K_{I(nonc)}$, mM	K_m, mM	MR, cm^3
I	$(CH_3)_3SiCH_2CH_2OH$	3.3	---	3.5	25.0
IA	$(CH_3)_3CCH_2CH_2OH$	7.5	19	5.3	19.6
IB	$(CH_3)_4N^+CH_2CH_2OHCl^-$	0.4	7.6	0.33	17.2
II	$(CH_3)_2S^+CH_2CH_2OHI^-$	0.4	13	0.33	16.4
III	$(CH_3)_3S^+I^-$	2.0	7.2	---	---
IV	$(CH_3)_3S^+OI^-$	1.3	---	---	---
V	$(CH_3)_3N^+O^-$	>>200	---	18	---
VI	$(CH_3)_2N^+(O^-)CH_2CH_2CH_2COCH_3$	14	---	18	16.4
VII	$CH_3S(O_2)CH_2CH_2OH$	8.7	100	6.2	13.9
VIIA	$CH_3S(O_2)CH_2CH_2OCOCH_3$	6.4	11	6.2	---
VIII	$(CH_3)_2SO_2$	28	260	---	---
IX	$(CH_3)_2SO$	25	---	---	14.1
X	$CH_3SCH_2CH_2OH$	40	---	15	13.3
XI	$CH_3CH_2CH_2CH_2OH$	47	---	13	10.3

[a] From Ref. 25. See Ref. 25 for details and references.

Table III

Reversible Inhibition by Pyridine Derivatives of Hydrolysis of AcCh by *Electrophorus* AcChE, pH 7.8[a]

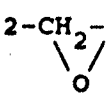
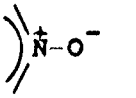
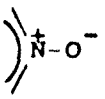
Substituent	pK_a	$\%AH^+$	$K_{i(com)}$ (mM)	$K_{i(nonc)}$ (mM)	$\Delta G_{(com)}$ kcal/mole
H–	5.25	---	8.1	---	---
H–[b]	5.25	0.06	12	110	-2.6
4-$(CH_3)_3C$–	5.3	---	1.3	---	---
4-$(CH_3)_3C$–[b]	5.3	0.06	3.7	13	-3.3
2-CH_2=CH–	4.98	0.15	1.4	10	-3.8
4-CH_2=CH–	5.62	0.07	2.0	6.6	-3.6
2- 	3.84	0.01	1.5	14	-3.8
3-CH_3CO–	---	---	2.4	2.4	-3.5
3-$BrCH_2CO$–	---	---	2.1	---	---
	0.79	---	11	63	-2.6
4-O_2N - 	---	---	2.1	2.7	---
4-O_2N–	---	---	2.0	2.8	---
2-CH_3O–	---	---	4.0	---	---
2-H_2N–	6.86	10	2.2	3.6	-3.6
3-H_2N–	5.98	1.5	0.82	??	-4.2
3-H_2N–[c]	5.98	0.1	10	11	-2.6
2-H_2N - 4-CH_3–	7.8	50	0.26	1.6	-4.8

Table III (Cont.)

Reversible Inhibition by Pyridine Derivatives of Hydrolysis of AcCh by Electrophorus AcChE, pH 7.8[a]

Substituent	pK_a	$\%AH^+$	$K_{i(com)}$ (mM)	$K_{i(nonc)}$ (mM)	$\Delta G_{(com)}$ kcal/mole
$2\text{-}H_2N$ - $4\text{-}CH_3$-[c]	7.8	5.9	1.1	6.0	-4.0
$2\text{-}(CH_3)_2N$-	6.99	13	0.08	1.3	-5.5
$3\text{-}(CH_3)_2N$-	6.46	4.4	0.10	0.82	-5.4
$3\text{-}(CH_3)_2N$-[c]	6.46	0.3	0.79	1.2	-4.2
$3\text{-}(CH_3)_2N$-[d]	6.46	48	0.67	0.25	-5.6
$4\text{-}(CH_3)_2N$-	9.7	99	0.05	0.12	-5.8
$\overset{+}{N}\text{-}CH_3$ - $3\text{-}(CH_3)_2N$-	---	(100)	0.02	0.06	-6.3
$\overset{+}{N}\text{-}CH_3$	---	(100)	0.16	0.20	-5.4

[a] Values at other pH's are noted.

[b] At pH 8.5.

[c] At pH 9.

[d] At pH 6.5.

Table IV

Noncompetitive Inhibition by Quaternary and Tertiary Compounds of Hydrolysis by *Electrophorus* AcChE of Substrates, $X\text{-}CH_2CH_2OCOCH_3$, of Varying Reactivity

No.	Inhibitor	$K_{i(com)}$ (mM)	Non-Competitive Binding Constants, $K_{i(nonc)}$, mM, pH 7.8				
			X-$(CH_3)_3N^+$-	X-$(C_2H_5)_2(n\text{-}C_4H_9)N^+$-	X-$(CH_3)_3C$-	X-$CH_3S(O_2)$-	X-CH_3CH_2-
I.A	$(CH_3)_4N^+$	2.9 ±0.6	20±10	11	5.2	2.8	3.4
IB	$(CH_3)_3NH^+$	4.5 ±4.5	1.6	6.1	1.2	22	18
IIA	$(CH_3)_3N^+CH_2CH_2OH$	0.84±0.27	9.1	4.2	1.7	2.2	2.7
IIB	$(CH_3)_2N^+HCH_2CH_2OH$	3.2 ±1.6	2.5	6.5	2.8	6.7	6.6
IIIA	$(CH_3)_3N^+CH_2CH_2OC_2H_5$	0.74±0.33	7.0	---	2.1	---	0.9
IIIB	$(CH_3)_2N^+HCH_2CH_2OC_2H_5$	3.8 ±3.3	0.40	---	0.9	---	2.0
IVA	$(CH_3)_3N^+(CH_2)_3COCH_3$	0.033	competitive				
IVB	$(CH_3)_2N^+H(CH_2)_3COCH_3$	1.2	2.5	---	3.6	---	8.0
VA	$(CH_3)_3N^+CH_2CH_2NHCOCH_3$	3.3	11	7.5	2.6	---	---
VB	$(CH_3)_2N^+HCH_2CH_2NHCOCH_3$	2.6	4.7	3.2	1.7	---	---
VIA	$(CH_3)_3CNH_3^+$	0.73	4.1	1.5	1.2	1.5	0.7
VIB	$(CH_3)_2HCNH_3^+$	2.1	16	---	1.2	5.8	5.9
	k_{cat} sec^{-1}		1.6×10^4	1.7×10^3	8.2×10^3	1.3×10^3	2.6×10^3
	K_m mM		0.27	0.13	1.5	6.8	13
	k_{cat}/K_m $M^{-1}sec^{-1}$		5.9×10^7	1.3×10^7	5.5×10^6	2.0×10^5	2.0×10^5

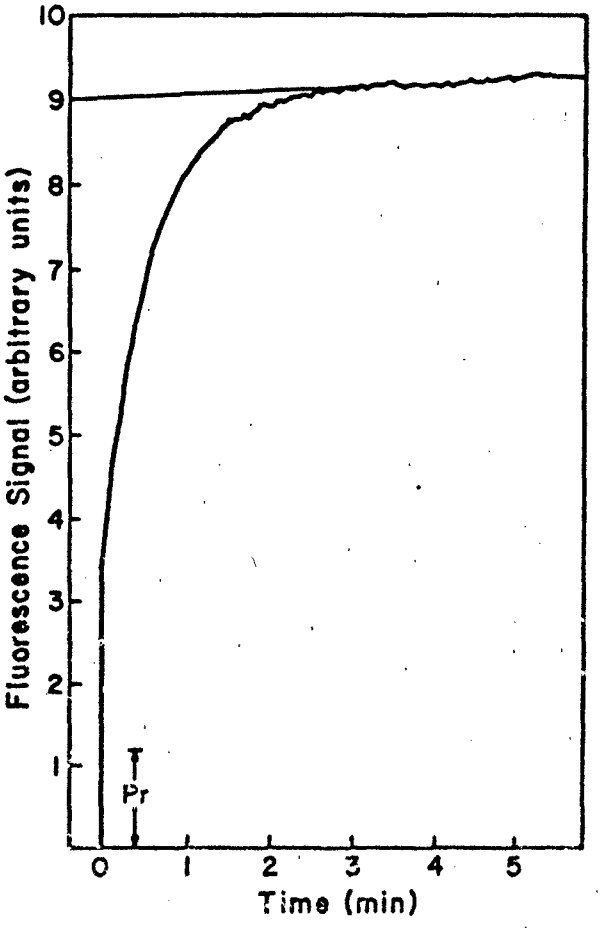


Figure 1. Titration of AcChE from T. nobiliana, with 4.75 μM M7CQ, pH 7.0, by burst release of M7HQ, observed by excitation at 400 nm and emission at 510 nm, indicating 12.8 nM AcChE; 10.2 μM, 70% active enzyme in the concentrate. The slow increase indicates decarbamylation, k about 1.5 x 10^{-4} sec^{-1}. The initial emission, indicated < Pr >, represents protein scatter.

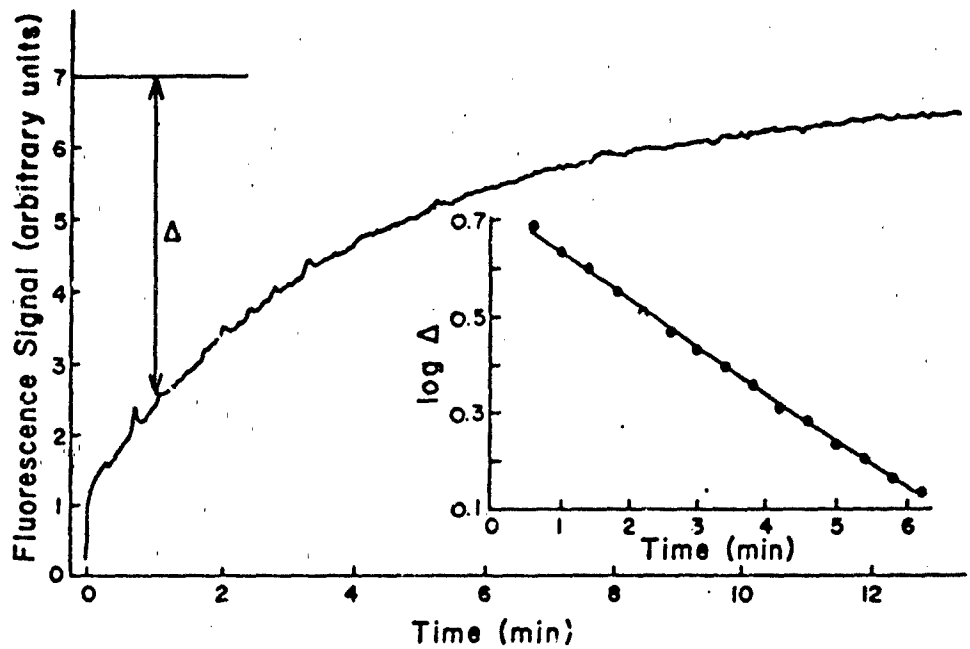


Figure 2. Titration of AcChE from T. nobiliana, with 0.475 μM M7CQ, (< K_i and > [E]), allowing observation of the carbamylation. The linear logarithmic insert indicates first order kinetics, k_{app} = 3.8 x 10^{-3} sec^{-1}; see text for further analysis.

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GLOSSARY OF ABBREVIATIONS

AcCh	Acetylcholine
AcChE	Acetylcholinesterase
AcSCh	Acetylthiocholine
BAc	n-Butyl acetate
$[^{14}C]$-BrPin	1-Bromo-2-$[^{14}C]$-pinacolone
$[^{14}C]$-Br_2Pin	1,1-Dibromo-2-$[^{14}C]$-pinacolone
DEC	Decamethonium
$[^{3}H]$-DFP	1,3-$[^{3}H]$-Diisopropyl fluorophosphate
DMAP	2-Dimethylaminopyridine
DMBAc	3,3-Dimethylbutyl acetate
DMCSCh	Dimethylcarbamylthiocholine
DMMP	3-Dimethylamino-N-methylpyridinium ion
EA	Acetylenzyme
EAI	Acetylenzyme bound to an inhibitor
EAS	Acetylenzyme bound to a substrate as an inhibitor
EBAAc	2-N,N-diethyl-N-butylammonioethyl acetate
ES	Enzyme-substrate complex
ESI	Enzyme-substrate complex bound to an inhibitor
ESS	Enzyme-substrate complex bound to another substrate as an inhibitor
GLC	Gas-liquid chromatography
IPTDZ	3-(3'-Iodo)phenyl-3-trifluoromethyldiazirine
M7CQ	N-Methyl-(7-dimethylcarbamoxy)quinolium ion
M7HQ	N-Methyl-7-hydroxyquinolinium ion
MR	Molar refraction

MBS	Methyl benzenesulfonate
MSEAc	Methylsulfonylethyl acetate
NMP	N-Methylpyridinium iodide
PTA	Phenyltrimethylammonium ion
PTDZ	3-Phenyl-3-trifluoromethyldiazirine
SDS-PAGE	Sodium dodecyl sulfate-polycarylamide gel electrophoresis
TAP	5-Trimethylammonio-2-pentanone
TFA	Trifluoroacetic acid
TMAAPh	3-Trimethylammonioacetophenone
TMAP	3-Trimethylammoniophenol